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November 22, 2005

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Title of Dissertation: "The regulation of lactogenic differentiation in mammary epithelial cells by Ras-dependent and -independent signal transduction"

Name of Candidate: Traci Galbaugh
Doctor of Philosophy Degree
6 January 2006

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OF DOCTOR OF PHILOSOPHY

Name of Student: Traci Galbaugh

Date of Examination: 6 January 2006

Time: 1:30

Place: Lecture Room C

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
Gabriela Dveksler, Ph.D. Department of Pathology Chairperson	✓	
Mary Lou Cutler, Ph.D. Department of Pathology Major Advisor	✓	
Philip Grimley, M.D. Department of Pathology Member	✓	
Elliot Kagan, M.D. Department of Pathology Member	✓	
Thomas Darling, MD, Ph.D. Department of Dermatology Member	✓	

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Traci Galbaugh
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ABSTRACT

Title of Dissertation: The regulation of lactogenic differentiation in mammary epithelial cells by Ras-dependent and –independent signal transduction.

Name, degree, year: Traci Galbaugh
Doctor of Philosophy
2005

Dissertation directed by: Dr. Mary Lou Cutler, Ph.D.
Associate Professor
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Epidermal growth factor (EGF) stimulation of mammary epithelial cells (MECs) inhibits differentiation and apoptosis. Excess activation of signaling pathways downstream of the EGF receptor has been directly linked to breast cancer development and chemotherapeutic resistance. Therefore, the dissection of these pathways in normal MECs is critical to the understanding of signaling events that impact breast cancer outcome. HC11 mouse MECs differentiate in response to lactogenic hormone resulting in expression of milk proteins including β-casein. Previous studies have shown that EGF blocks differentiation through activation of the Ras/Mek/Erk and the PI-3-K pathway. Therefore, specific chemical inhibitors of signal transduction pathways, activated Ras (RasV12), dominant negative-Ras (DN-RasN17), dominant negative-Akt adenovirus (DN-Akt-adeno) and a conditionally active-Akt (CA-Akt) were used to analyze the role of the Ras and PI-3-K pathway in blocking HC11 lactogenic differentiation.

Activated RasV12 expression resulted in reduced tyrosine phosphorylation of Stat5 and β-casein expression in response to prolactin. However, the expression of DN-RasN17 enhanced Stat5 tyrosine phosphorylation, Stat5 DNA binding and β-casein

transcription. The expression of DN-RasN17 also blocked the activation of the Mek/Erk pathway by EGF and prevented the block of lactogenic differentiation induced by EGF. Stimulation of HC11 cells with prolactin resulted in the association of the SHP2 phosphatase with Stat5, and this association was inhibited by DN-RasN17 expression.

Furthermore, the expression CA-Akt blocked lactogenic differentiation of HC11 cells. In contrast, treatment with LY294002 or infection with DN-Akt-adeno enhanced β-casein transcription and rescued β-casein promotor driven luciferase activity in the presence of EGF, demonstrating that the EGF block of HC11 lactogenic differentiation is, in part, dependent on PI-3-K/Akt. Moreover, the inhibition of either PI-3-K (LY294002) or mTOR (Rapamycin) abolished the activation of p70S6 Kinase (p70S6K) by EGF in HC11 cells, suggesting that PI-3-K signaling via p70S6K contributes to the EGF block of lactogenic differentiation. Additional investigation determined that EGF activates p70S6K resulting in the phosphorylation of RPS6, eIF4E and 4E-BP1 via PI-3-K/Akt/mTOR dependent mechanisms.

In conclusion, these results demonstrate that in HC11 cells, DN-Ras inhibits the Mek/Erk pathway and enhances lactogenic hormone-induced differentiation, in part by inhibiting the association of the SHP2 phosphatase with Stat5. In addition, this data demonstrates that the EGF-induced activation of PI-3-K stimulates the Akt/mTOR/p70S6K pathway to influence the regulation of lactogenic hormone-induced differentiation.

The regulation of lactogenic differentiation in mammary epithelial cells by Ras-dependent and –independent signal transduction

By

Traci Galbaugh

Dissertation submitted to the Faculty of the Graduate Degree Program in Pathology of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy
2005

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The past five and a half years of graduate study at USUHS has proven to be a challenging, life changing venture that I could not have accomplished alone. I wish to express my appreciation to the members of my dissertation committee: Dr. Gabriela Dveksler, Chair, Dr. Phil Grimley, Dr. Elliot Kagan, and Dr. Thomas Darling. I would also like to express my gratitude to Dr. Mary Lou Cutler, my dissertation advisor. She has been a supportive mentor and I am grateful for the wonderful learning opportunities she has provided. Treasa Chopp provided technical advice on laboratory techniques; I am indebted to her for her teaching and assistance. In addition, I would like to thank several of my fellow classmates, Katie Daddario, Julie Wu, Tara Romanczyk, David Kuch, Mark Sercovich and Bethanie Morrison for their advice, support, and friendship. Finally, I wish to thank my partner, Dawn Dolby, who has been my backbone throughout this endeavor.

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GLOSSARY

4E-BP1: eIF4E-binding protein 1

CA: constitutively active

CIS: cytokine-inducible SH2 protein

DIP: dexamethasone, insulin and prolactin

DN: dominant negative

EGF: epidermal growth factor

eIF4E: elongation initiation factor 4E

GSK3β: glycogen synthase kinase 3 beta

Jak2: janus kinase 2

JNK: jun N-terminal kinase

LY294002: PI-3-K inhibitor

MECs: mammary epithelial cells

mTOR: mammalian target of Rapamycin

NFκB: nuclear factor kB

OPN: osteopontin

p38K: p38 kinase

p70S6K: p70S6 Kinase

PD98059: Mek inhibitor

PDK1: 3-Phosphoinositide-dependent protein kinase-1

Phospho: phosphorylated

PI-3-K: phosphatidylinositol-3-kinase

PIP: prolactin inducible protein

PIP3: phosphatidylinositol-3, 4, 5-triphosphate

PRL: prolactin

RasV12: activated Ras

Rapamycin: mTOR inhibitor

RPS6: ribosomal protein S6

RTK: receptor tyrosine kinase

SB203850: p38 kinase inhibitor

Ser: serine

SHP2: SH2 protein tyrosine phosphatase

Stat5: signal transducer and activator of transcription 5

SOCS: suppressor of cytokine signaling

TGFα: transforming growth factor α

Thr: threonine

TRE: tet-responsive element

Tyr: tyrosine

WAP: whey acidic protein

PART ONE

INTRODUCTION

INTRODUCTION

Epidemiology

Breast cancer incidence in women has increased from one in twenty in 1960 to one in eight in 2005 and is the most commonly occurring cancer in women. In 2005, approximately 212,000 women in the United States (US) and one million worldwide will be diagnosed with invasive breast cancer, and 40,000 in the US alone will die from the disease (ACS, 2005). Based on epidemiological studies, risk factors for developing breast cancer include: age, nullparity, number of reproductive events, geographical location, exogenous hormone intake, lifestyle, and family history of disease and genetic background (Table 1) (Simpson, 2002; Dumitrescu, 2005).

Breast cancer incidence is very low before the age of 25; however, when breast tumors do occur in younger women, these tend to be extremely aggressive and are associated with a particularly high mortality rate. Between 25 and menopause, breast cancer incidence rates double every ten years, suggesting a role for hormone production in breast cancer development (ACS, 2005; McPherson, 2000; Dumitrescu, 2005). Post-menopausal breast cancer incidence continues to rise, peaking between 65 and 75 years for women in the US (McPherson, 2000). Pre-menopausal tumors tend to be more aggressive, as well as more resistant to chemotherapy, than post-menopausal tumors (Veronesi, 2005). Age at menarche and menopause are also associated with variable degrees of breast cancer risk. Early menarche and/or late menopause increase breast cancer risk (Dumitrescu, 2005). Menarche at 15 years, the high end of the normal range, is associated with a 30% lower risk of breast cancer than menarche at 11 years, the low end of the normal range (McPherson 2000; Colditz, 2005). Menopause at 45 years, the

low end of the normal range, carries a 44% lower risk of breast cancer than menopause at 55 years (Colditz, 2005).

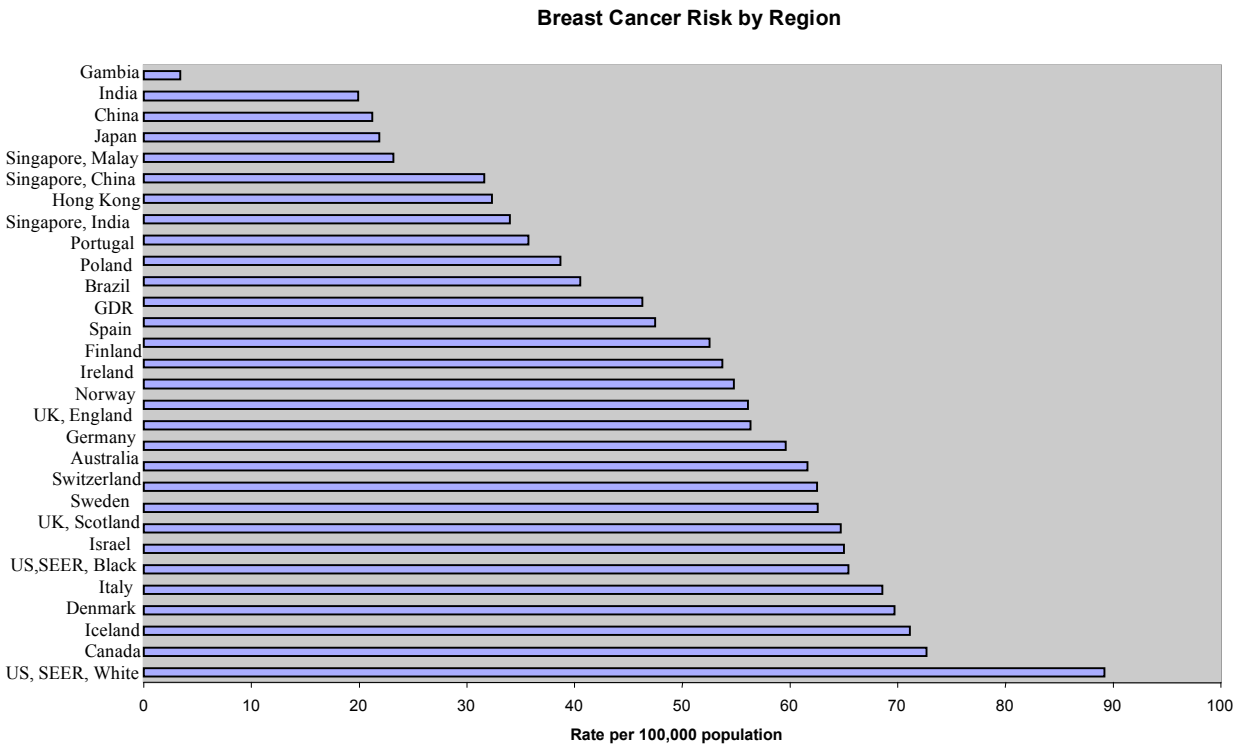
Table 1: Adapted from Veronesi, et al. *Breast Cancer.* Lancet, 2005. **365** (9472): p1727-41. **Breast cancer risk**

	High Risk Group
Age	Elderly
Geographic location	Developed countries
Age at menarche	Before age 11
Age at menopause	After age 54
Age at first full term pregnancy	Before age 20
Family history	Breast cancer in first degree relative
Body mass index	
Pre-menopausal	High body mass index
Post-menopausal	High body mass index
Alcohol consumption	2 or more drinks a day
Exposure to ionizing radiation	Abnormal exposure before puberty
Use of exogenous hormones	
Oral contraceptives	Current users
Hormone-replacement therapy	Current users

Surgically induced menopause before age 35 significantly reduces a woman's risk for breast cancer (Dumitrescu, 2005). A woman's age at first pregnancy and number of full-term pregnancies are also identified breast cancer risk factors. Early age at first full-term pregnancy (less than 20 years) decreases a woman's risk of breast cancer by 50% (Dumitrescu, 2005). Young age at second pregnancy as well as high parity also seem to reduce the risk of breast cancer before age 35 (Veronesi, 2005). Nullparity is one of the leading risks of breast cancer (Simpson, 2002).

Some epidemiological data suggests an association between geographical location and breast cancer incidence. The US and Europe have the highest percentages of women diagnosed with breast cancer. Asian countries, particularly those in the Far East, have the lowest rate of breast cancer. One study comparing the incidence of breast cancer in Japanese women living in Japan to those migrating to Hawaii and San Francisco led to the suggestion that environmental factors such as diet may outweigh genetic ones. While other types of cancer had higher incidences in Asian women, the breast cancer rate in Japanese women who migrated to the US was similar to that of American, not Japanese, women (McPherson, 2000). Different from most other diseases, women with a higher socioeconomic status (SES) are at a slightly higher risk for breast cancer than women that have a low SES (Dumitrescu, 2005). Also, in the US, Caucasian women have higher breast cancer incidence and mortality rates than women of any other ethnic group (Table 2).

Table 2: Adapted from McPherson, et al., *ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics.* BML, 2000. **321** (7261): p624-8.



Exogenous post-menopausal hormone use of 10 or more years also increases risk for breast cancer development. Several studies show that hormone-replacement therapy (HRT) increases breast density, a clinical finding directly linked to breast cancer development (Dumitrescu, 2005; McPherson 2000). The use of oral contraceptives before the age of 20 increases a woman's risk of breast cancer (Dumitrescu, 2005). Data from a Collaborative Group on Hormonal Factors study reported an association between oral contraceptive use and statistically higher risk of breast cancer. Upon cessation of oral contraceptive use, breast cancer risk decreases (Lancet, 1996).

Women who have been exposed to ionizing radiation, especially prior to puberty, have an exposure dose-dependent increased risk of developing breast cancer (Veronesi, 2005; Dumitrescu, 2005). While an association between pesticide exposure and increased breast cancer incidence has not been established (Veronesi, 2005), dietary risk factors suggest that agricultural practices should not be totally excluded from consideration. For example, natural and synthetic carcinogens are frequently identified in food and water supplies. On the other hand, some foodstuffs, such as fresh fruits and vegetables, contain high levels of "anti-carcinogens" (anti-oxidants). High pre-menopausal animal fat intake has been associated with a higher risk of breast cancer (Veronesi, 2005), and diets rich in sources of anti-oxidants seem to decrease breast cancer risk (Dumitrescu, 2005). Higher levels of physical activity, especially during adolescence and young adulthood (12-24 years), also seems to lower a woman's risk of breast cancer, probably by delaying menarche and altering hormone levels (Dumitrescu, 2005). Obesity in post-menopausal women is associated with an increased risk of developing breast cancer, even after adjusting for confounding effects of physical

activity, possibly as the result of endogenous estrogen sequestration by adipose tissue (Dumitrescu, 2005). Other lifestyle choices associated with an increased incidence of mammary tumors include alcohol consumption and cigarette smoking. Women who consume 2 or more drinks a day, whether pre- or post-menopausal, are more likely to develop breast cancer than those who do not. In addition to its well-known link to lung cancer, smoking has also been shown to be a risk factor for developing breast cancer.

A family history of breast cancer, particularly one involving first degree relatives, *i.e.,* mother, daughter or sibling, increases a woman's risk of breast cancer (McPherson 2000); one out of nine women diagnosed with breast cancer has an affected mother, sister, or daughter (Veronesi, 2005). Members of families with histories of breast cancer also show higher than normal rates of ovarian, colon, and prostate cancers (McPherson, 2005). Inherited loss-of-function mutations in tumor suppressor genes, e.g., those encoding the breast cancer susceptibility gene 1 and 2 (BRCA1 and 2), p53, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) proteins, are frequently involved in these familial cases and account for 5-10% of all breast cancers (McPherson, 2000; Dumitrescu, 2005). However, somatic mutation of these or other tumor promoter and suppressor genes are undoubtedly involved in disease progression in most, if not all, cases.

Risk factor identification provides clues for elucidating physiological and molecular pathogenetic mechanisms. For example, physiological mechanisms for explaining one epidemiological clue that early and numerous full-term pregnancies provide some degree of protection from breast cancer development, include: (1) mammary epithelial cells are fully differentiated during lactation and then undergo

apoptosis during involution allowing for any damaged DNA to be eliminated from the pool of proliferating epithelial cells, and (2) the circulating hormone and growth factor changes during pregnancy result in down-regulation of key contributors to breast cancer development, *e.g.,* estrogen and epidermal growth factor (Colditz, 2005; Dumitrescu, 2005). Epidemiology provides only clues, however. A thorough appreciation for the physiological and molecular pathogenetic mechanisms underlying breast cancer development and progression requires an equally thorough understanding of the structure and development of the functional and dysfunctional mammary gland.

Carcinogenesis is usually a multi-step process involving aberrant regulation of multiple cellular processes and/or signaling pathways. In becoming cancerous, a cell generally devises methods for averting numerous molecular, cellular, and systemic protective mechanisms (Hahn, 2002). Understanding the regulation of and interactions among these defenses during the normal cell life cycle, as well as at different stages in the transformation process, is of utmost importance for developing effective anti-cancer therapeutic regimens. Molecular dissection of the cellular pathways and processes implicated in tumorigenesis, particularly those associated with cell proliferation, differentiation, and apoptosis, is, therefore, a high priority in cancer research.

Epidermal growth factor

Epidermal growth factor (EGF) is required for normal mammary epithelial proliferation and functions through binding to the extracellular domains of EGF receptor (EGFR)/ErbB tyrosine kinase family members, which include EGFR/ErbB1 and ErbB4 proteins (Lichtner, 2003; LeVea, 2004; Parmar, 2004). ErbB2/neu and ErbB3 are

additional family members; however, ErbB2 does not bind ligand and ErbB3 possesses no inducible tyrosine kinase activity (Danielsen, 2002; Earp, 1995). Ligand binding causes receptor homo- or heterodimerization, the specificity of which demonstrates some degree of ligand dependence (LeVea, 2004). In addition to extracellular ligand-binding and intracellular tyrosine-containing domains, these type I receptor tyrosine kinases (RTKs) possess highly homologous intracellular kinase domains capable of phosphorylating tyrosine residues on intracellular signaling adaptor proteins and transmembrane domains involved in mediating interactions between the receptors and the intracellular domains (Normanno, 2003). Dimerization of the receptor results in its tyrosine kinase activation and in ATP-dependent ErbB tyrosine phosphorylation. These phosphotyrosine residues enable receptor interaction with signaling proteins that contain src homology-2 (SH2) or phosphotyrosine binding (PTB) domains (Navolanic, 2003).

While EGF is the only EGFR/ErbB ligand capable of inducing HC11 mouse mammary epithelial cell (MEC) competency (Beerli, 1996), EGF is not the only natural ligand for EGFR/ErbB family members. One class of EGFR/ErbB stimulators in addition to EGF, termed EGF-like, includes heparin binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGF-α), betacellulin (BTC), amphiregulin (AR), tomoregulin (TR), and epiregulin (EP) (Navolanic, 2003; Normanno, 2003). With the exception of TR, these have all been demonstrated to bind EGFR/ErbB1. HB-EGF, BTC, and EP are also capable of binding ErbB4, as are TR and the second class of EGFR/ErbB stimulators, the neuregulins (NRGs)/ heregulins/neu differentiation factors. This class consists of NRG1α, NRG1β, NRG2α, NRG2β, NRG3, and NRG4; in addition to ErbB4, neuregulins also associate with ErbB3 (Navolanic, 2003). Upon over-

expression, EGFR/ErbB becomes constitutively active, which can result in cellular transformation through constitutive cell cycle progression- and cell survival-promoting signaling pathway activation (Parmar, 2004; Navolanic, 2003). Up to 30% of all breast cancer tumors over-express one or more members of the EGF/ErbB receptor family and have been common targets for breast cancer therapy (LeVea, 2004; Riese, 1998; Danielsen, 2002; Stern, 2003).

Normal MECs possess multiple mechanisms for tightly regulating EGFR/ErbB family members. A RING type E3 ubiquitin ligase, c-Cbl, is one of the most important and well-studied EGFR/ErbB regulators. Autophosphorlyation of EGFR/ErbB at a lysosomal targeting motif recruits c-Cbl via the latter's SH2 domain, and its RING domain catalyzes the addition of poly-ubiquitin chains to RTKs, thereby targeting EGFR for lysosomal degradation (Shtiegman, 2003; Ohta, 2004). Interestingly, ErbB2 is resistant to c-Cbl-mediated degradation. c-Cbl is unable to associate with EGFR while complexed to ErbB2 because of ErbB2's inability to phosphorylate the c-Cbl-binding site on EGFR/ErbB (Ohta, 2004). Therapeutic antibodies that target ErbB2 are, therefore, hypothesized to allow c-Cbl recruitment and c-Cbl-mediated degradation.

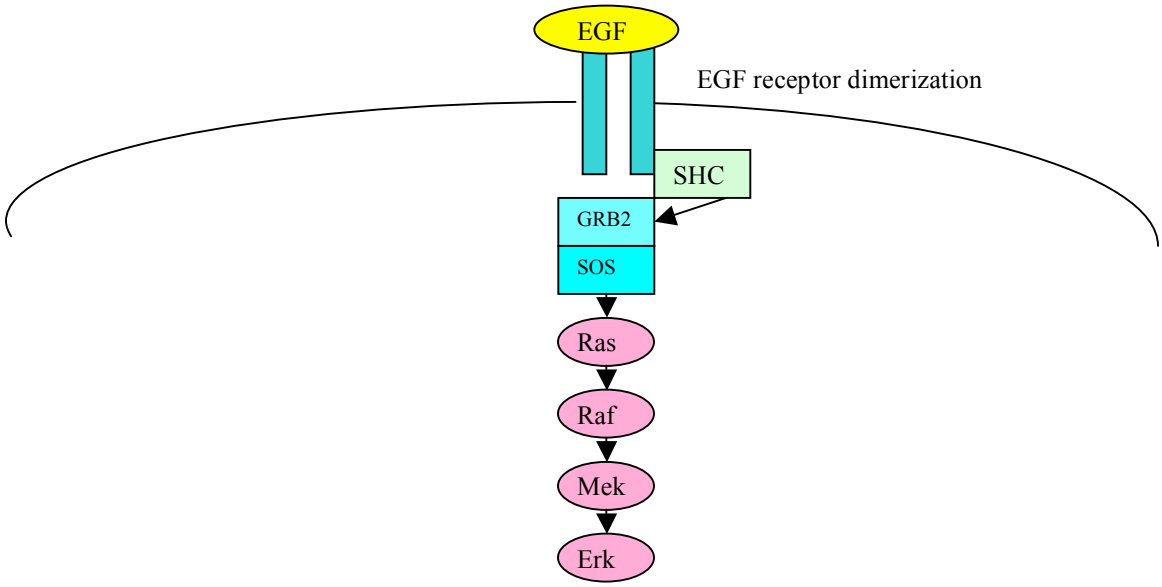
In cell culture, high cell density results in down-regulation of EGFR/ErbB and signaling; EGFR/ErbB autophosphorylation is suppressed in cells experiencing contact inhibition as compared to cells cultured at low density (LeVea, 2004). Akt activation is concurrently reduced in high-density cells. Deregulation of EGF/ErbB and Akt signaling in response to contact inhibition has, therefore, been proposed to represent a mechanism for cellular transformation and to contribute to the chemotherapeutic resistance of tumors (LeVea, 2004).

Excessive activation of signaling pathways downstream of EGFR has also been directly linked to breast cancer development and chemotherapeutic resistance (Navolanic, 2003). The two main signaling pathways activated by EGF stimulation, both of which have been implicated in EGF-mediated cellular transformation, are the Ras/Mek/Erk and the phosphatidylinositol 3-kinase (PI-3-K) pathways (LeVea, 2004). In the first of these pathways, EGFR cytoplasmic domain tyrosine residue autophosphorylation (Figure 1) results in its recruitment of and binding to the constitutively-associated growth factor receptor binding protein 2 (Grb2)-son of sevenless (SOS) complex. SOS, a guanine nucleotide exchange factor, then converts inactive Ras-GDP to active Ras-GTP. Raf, a Mek kinase, is then translocated to the plasma membrane and undergoes Ras-mediated activation. Raf, in turn, activates Mek via serine phosphorylation, and Mek activates Erk by threonine and tyrosine phosphorylation (LeVea, 2004). EGF-induced Ras/Mek/Erk activation inhibits hormone-induced lactogenic differentiation (Hynes, 1990).

EGF- and Ras-associated mitogenic signal transduction pathways are frequently activated in breast carcinoma, and these inhibit mammary differentiation and apoptosis. Data from our laboratory suggest that HC11 MECs, which differentiate and synthesize β-casein following growth to confluency and stimulation with lactogenic hormones, experience EGF-mediated suppression of differentiation. This suppression is due to Ras-induced activation of the Mek/Erk cascade and to Ras-independent PI-3-K pathway activation (Cerrito, 2004). Chemical inhibition of either Mek/Erk or PI-3-K signaling prevented EGF-mediated inhibition of prolactin-induced HC11 cellular differentiation. EGF stimulation results in the activation of Ras and blocks lactogenic differentiation. The results from β-casein reporter, Stat5 phosphorylation and DNA-binding assays indicate

that induced expression of a constitutively active-Ras also inhibited lactogenic differentiation. On the other hand, expression of a dominant negative-Ras allowed differentiation and prevented Mek/Erk pathway activation without affecting the activation status of Akt/PKB, a PI-3-K pathway effector molecule.

Figure 1; Adapted from Navolanic, et al: *Int. J.Onc*. 22: 237-252, 2003: Dimerization of EGFR induces the Raf/Mek/Erk kinase cascade. Upon dimerization of the EGF receptors by EGF, a kinase cascade is induced, which transmits information from the cell membrane to transcript factors that control gene expression in the nucleus. Upon EGF mediated EGR dimerization and receptor phosphorylation, Shc binds the receptor, which further transmits the signal to GRB2, Sos and Ras. Ras in turn activates Raf, which leads to the induction of the kinase cascade.

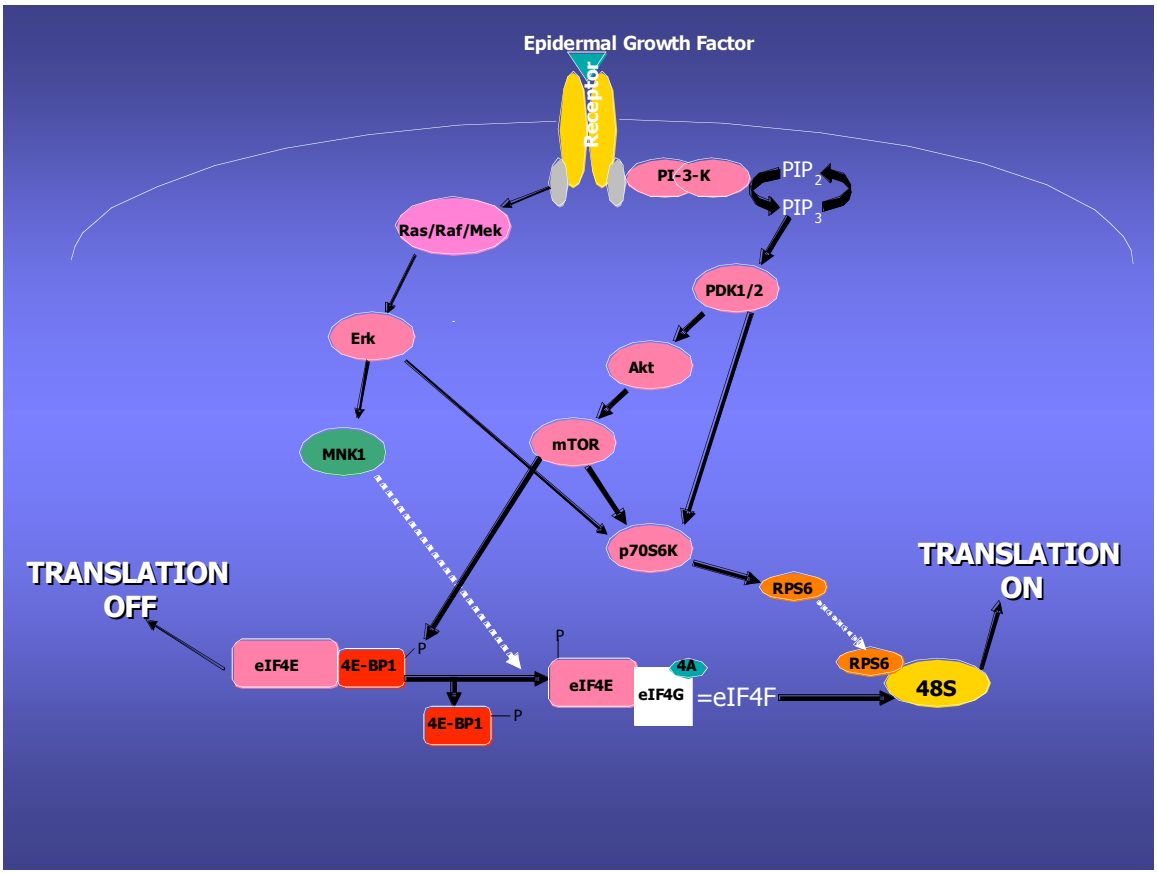


Phosphatidylinositol-3-kinase

The other major EGF-dependent pathway implicated in cell cycle progression, cell survival and cellular transformation is the PI-3-K pathway (Figure 2). In broad terms, the cellular pathways implicated in tumorigenesis can be divided into two categories: those in which alterations bestow a proliferative advantage upon the transformed cell and those in which changes allow the transformed cell to evade apoptosis (Fresno, 2004). The PI-3-K pathway is particularly important in tumorigenesis as aberrant PI-3-K activation has been demonstrated to promote both proliferation and survival of transformed cells, including those exhibiting EGF-dependent transformation (Fresno, 2004). The deregulation of many PI-3-K pathway components has recently been linked to a number of human malignancies (Vivanco, 2002; Fresno, 2004); aberrant activation of Akt, for example, have been found in breast, ovarian, colon and thyroid cancers (Lou, 2003; Vivanco, 2002).

Gershtein *et al.* were first to correlate PI-3-K protein expression levels with breast cancer development. Biopsy analysis revealed that PI-3-K over-expression was present in tumor tissue, but not the surrounding normal tissue in 28 of 33 breast cancer patient samples. Comparisons of PI-3-K expression levels in tumor tissue from pre-menopausal versus post-menopausal patients demonstrated that the pre-menopausal tumors, noted for their associated poor prognosis and chemoresistance, expressed significantly higher levels of PI-3-K (Gershtein, 1999). This supports the idea that women with breast tumors that over-express PI-3-K have a poorer prognosis and are more likely to develop chemotherapeutic resistance than those with low-level PI-3-K-expressing breast tumors.

Figure 2; Adapted from www.cellsignaling.com. Model for the regulation of the PI-3-K/Akt signaling pathway. Kinase activation is the consequence of translocation of PI-3-K from the cytosol, in which PI-3-K is thought to be inactive, to the plasma membrane, providing access to its activators and substrate. The p85-p110 complex is recruited to the intracellular domain of the autophosphorylated growth factor receptor. PI-3-K activation is the result of a conformational change induced by p85--EGFR binding via p85 SH2 domains and receptor phosphotyrosine residues. PI-3-K-mediated generation of PIP3 directs the membrane localization of phosphatidylinositol-dependent kinase 1 (PDK1) through PDK1's pleckstrin homology domain. PDK1 activation through autophosphorylation ensues with subsequent PDK1-mediated phosphorylation activation of downstream PI-3-K pathway signal transducers and effectors, *e.g.,* Akt/PKB. Akt activates mTOR and two major mTOR effectors have been identified. The first, p70S6K, is activated by mTOR and regulates ribosomal biogenesis and cellular translation capacity through promoting selective translation of mRNAs containing 5' oligopyrimidine tracts. mTOR-mediated phosphorylation of the second, 4E-BP1, facilitates cap-dependent translation by preventing association of this inhibitor and its eukaryotic translation initiation factor 4E (eIF4E) binding partner.



The Yu laboratory subsequently examined the relationship between breast tumor progression and the, presumably PI-3-K-dependent, phosphorylation of Akt and activation of downstream Akt/mammalian target of Rapapmycin (mTOR) pathway effectors. This group demonstrated that the phosphorylation of Akt/mTOR increased progressively from normal breast epithelia to hyperplasia and abnormal hyperplasia to tumor invasion, suggesting that PI-3-K activity levels directly correlate with the degree of tumor progression (Zhou, 2004). The Gershtein *et al.* and Yu *et al.* findings not only demonstrate the prominence of PI-3-K expression in breast cancer and its utility as prognostic indicator, but also lead to the speculation that ligand-induced and/or constitutive PI-3-K pathway activation might represent an important step in breast tumorigenesis.

The PI-3-Ks comprise a family of ubiquitously-expressed lipid kinases, one of which was first purified and cloned as a heterodimeric complex consisting of an 110kDa catalytic subunit and an 85kDa regulatory/adaptor subunit (Cantley, 2002; Vivanco 2002). Three isoforms of the catalytic subunit, designated p110α,β and γ, and seven adaptor proteins generated by the alternative splicing of three genes (*p85α, p85β* and *p55γ*), have been identified (Fresno, 2004). The heterodimers formed among these catalytic and adaptor subunits are termed class 1 PI-3-Ks. These enzymes phosphorylate the inositol ring 3'-OH group on phosphatidylinositol-4, 5-bisphosphate to generate the second messenger, phosphatidylinositol-3, 4, 5-triphosphate (PIP3) (Fruman, 1998). The regulatory subunit keeps the catalytic subunit in a low activity state in dormant cells (Cantley, 2002). Kinase activation is the consequence of translocation of PI-3-K from

the cytosol, in which PI-3-K is thought to be inactive, to the plasma membrane, providing access to its activators and substrate (Vivanco, 2002).

In epithelial cells, two mechanisms for PI-3-K activation have been identified to date: via binding of the p110 subunit to activated Ras and through binding of p85 subunit to the EGFR. In this latter mechanism, the p85-p110 complex is recruited to the intracellular domain of the autophosphorylated growth factor receptor. PI-3-K activation is the result of a conformational change induced by p85--EGFR binding via p85 SH2 domains and receptor phosphotyrosine residues (Cantley, 2002; Navolanic, 2003). Our laboratory has demonstrated that EGF-dependent PI-3-K activation is independent of Ras activation in HC11 mouse MECs: blocking Ras-dependent prevention of MEC lactogenic differentiation did not affect EGF-stimulated PI-3-K activation (Cerrito, 2004). EGF has, however, been shown to inhibit HC11 MEC differentiation via PI-3-K activation (Hynes, 1990; De Santis, 1997; Cerrito, 2004). EGF also inhibits hormone-induced differentiation via the activation of PI-3-K and its downstream effectors involved in cellular growth, proliferation and survival.

PI-3-K promotes cellular survival through several downstream effector proteins, the activation and functions of which demonstrate some degree of cell-type dependence. PI-3-K-mediated generation of PIP3 directs the membrane localization of phosphatidylinositol-dependent kinase 1 (PDK1) through PDK1's pleckstrin homology domain. PDK1 activation through autophosphorylation ensues with subsequent PDK1-mediated phosphorylation activation of downstream PI-3-K pathway signal transducers and effectors, *e.g.,* Akt/PKB, possibly Src and Caveolin 1 (Xie, 2003).

Although Akt/PKB-independent effects of PI-3-K activation have been described, Akt/PKB is the most prominent cellular mediator of PI-3-K activation-induced responses. The Akt protein family is comprised of three members, Akt 1, 2, 3/PKB1, 2, 3, all of which are highly similar (Fresno, 2004). Akt is predominantly activated by PI-3-K, but understanding of its cell-type specific activation and regulation is incomplete. In EGF-treated MECs, blocking the PI-3-K pathway with LY294002 completely abolishes Akt activation (Galbaugh, thesis work), suggesting Akt activation is primarily activated through PI-3-K in MECs. In most cell types, Akt can be phosphorylated at two different sites, threonine (Thr) 308 and serine (Ser) 473. PDK1 phosphorylates Akt at Thr308, and this is thought to be sufficient for the activation of Akt (Vivanco, 2002). However, maximal activation of Akt requires phosphorylation of Ser473 by an as-yet incompletely characterized kinase designated PDK2 (Vivanco, 2002). Recent studies suggest that Ser473 phosphorylation precedes that of Thr308 and that Ser473 phosphorylation is crucial for the recognition and activation by PDK1 (Sarbassov, 2005).

At least thirteen Akt substrates, including mTOR, glycogen synthase kinase-3β (GSK3β), p70 S6 Kinase (p70S6K), cyclin D1, caspase 9, Bad, nuclear factor κB (NFκB) and the forkhead receptor 1 (FKHR1), have been identified thus far, most of which are inhibited by Akt-mediated phosphorylation. PI-3-K regulates a number of biological processes such as cellular growth (increase in cell size), proliferation (increase in cell number), differentiation and survival by either activating or repressing multiple factors and co-factors (Vivanco, 2002; Shen, 2003).

The first of these biological processes, cellular growth, refers to increased cell size as the result of macromolecule synthesis. Akt activates mTOR, a PI-3-K-like

serine/threonine kinase and a key controller of cell growth via coordination of protein synthesis with nutrient availability. Two major mTOR effectors have been identified. The first, p70S6K, is activated by mTOR and regulates ribosomal biogenesis and cellular translation capacity through promoting selective translation of mRNAs containing 5' oligopyrimidine tracts. mTOR-mediated phosphorylation of the second, 4E-BP1, facilitates cap-dependent translation by preventing association of this inhibitor and its eukaryotic translation initiation factor 4E (eIF4E) binding partner. Akt directly activates mTOR, and the PI-3-K inhibitor, LY294002, can inhibit this activation. While Akt may not be the only activator of mTOR in some or all cell types, experiments from our laboratory using LY294002 and the mTOR-specific inhibitor, rapamycin, demonstrated that, in HC11 cells, Akt mediates mTOR and subsequent p70S6K activation.

Cellular proliferation refers to an increase in cell number as the result of cell division, a process controlled by regulators of cell cycle progression. Cell cycle phase transitions are regulated by cyclin-dependent kinases (Cdks), the activation of which is subject to strict regulation. As the Cdk designation implies, association with a cyclin partner is required for Cdk activation. Cdk activity is inhibited via association with endogenous cyclin-dependent kinase inhibitors (CKIs); depending on the CKI involved, this may occur either in the presence or absence of cyclin-Cdk complex formation. Another level of Cdk control is through direct phosphorylation and dephosphorylation of Cdk amino acid residues, both of which may positively or negatively influence Cdk activity. PI-3-K/Akt activity regulates cyclin D1. This binding partner of G1 Cdks, *e.g.* Cdk4, is particularly important in regulating the G1/S phase transition (Vivanco, 2002). Active Akt prevents cyclin D1s degradation through inhibitory phosphorylation of one of

its targets, GSK3β (Cantley, 2002). Experiments employing dominant negative- and RNA interference (RNAi)-mediated, as well as pharmacological, inhibition of PI-3-K/Akt activity have demonstrated that cyclin D1 accumulation resulting from Akt activation and GSK3β suppression causes cells to remain in their proliferative state, blocking cell cycle arrest and differentiation (Fry, 2001). Our data demonstrate that in MEC's cyclin D1 expression is EGF-dependent. These data also suggest that this expression may be PI-3-K-dependent as LY294002 significantly decreased EGF's ability to stimulate cyclin D1 expression.

Due to unique maturation characteristics in each of the broad variety of cell types, cellular differentiation regulation, including PI-3-K's involvement therein, is poorly understood. PI-3-K's role in differentiation appears to vary greatly from one cell type to another: PI-3-K promotes growth arrest in keratinocytes and has been demonstrated to be required for differentiation of both these and adipocytes (Calautti, 2005; Aubin, 2005). In intestinal epithelial cells, however, PI-3-K mediates proliferative signals and inhibits differentiation (Sheng, 2003). Data from our laboratory demonstrate that EGF-mediated PI-3-K activation inhibits the differentiation of HC11 MECs normally induced by exogenous DIP [dexamethsone (DEX), insulin and prolactin (PRL)], a combination that, along with EGF, mimics the *in vivo* hormone and growth factor requirements for MEC differentiation post-parturition. MEC differentiation, in turn, permits β-casein synthesis and, ultimately, milk production (Kelly, 2002).

Promotion of cell survival by preventing apoptosis is the most important function of the PI-3-K/Akt pathway, and PI-3-K appears to be required for cellular survival of both cycling and non-cycling cells (Lynch, 2002). In normal cells, programmed cell

death controls excessive proliferation. Most cancer cells, however, have devised methods for evading apoptosis, thereby lengthening their lifespan. The most common strategy by which cancer cells avoid apoptosis appears to be via Akt activation: PI-3-K/Akt constitutive activity correlates closely with resistance to apoptosis in cancer cells (Cantley, 2002; Fry, 2001; Vivanco, 2002). PI-3-K pathway activation results in inactivating phosphorylation of many pro-apoptotic proteins and activating phosphorylation of many anti-apoptotic proteins. For example, Akt phosphorylates the pro-apoptotic Bad protein, preventing Bad-mediated inactivation of the anti-apoptotic Bcl-X_L. Akt also phosphorylates and inactivates caspase 9, a cell death-promoting protease. Phosphorylation of another Akt target, FKHR1, prevents its nuclear translocation and FKHR1-mediated transcription of several pro-apoptotic proteins. Akt also influences cell survival indirectly by activating NFκB. Akt-induced phosphorylation of IκB kinase (IKK) leads to its activation and subsequent inhibitory phosphorylation of the NFκB inhibitor, IκB. IκB phosphorylation results in its proteasomal degradation and in NFκB release and nuclear translocation.

Dephosphorylation of the second messenger, PIP3, is critical for down-regulating the activity of the PI-3-K pathway when appropriate. Two major lipid phosphatases, PTEN and SHIP, dephosphorylate PIP3, thereby quenching PI-3-K pathway signaling (Cantley, 2002; Cantley, 1999). The importance of PTEN in preventing prolonged activation of this pathway is exemplified by the pathophysiology of Cowden's syndrome, the result of specific germline mutations in the gene encoding PTEN. Cowden's syndrome patients are prone to malignant tumor development, particularly breast carcinomas (Fresno, 2004). Also, PTEN gene somatic mutations are among the most

commonly identified in a broad range of cancers, including those of the prostate, breast, and lung, as well as in central nervous system tumors such as glioblastomas (Luo, 2003).

Because of PI-3-K/Akt signaling involvement in a broad range of biological processes and the high degree of correlation between PI-3-K/Akt pathway deregulation in a similarly broad range of cancers, including those involving mammary epithelium (Fry, 2001), elucidating the roles of PI-3-K signaling in the normal MEC life cycle is a necessary first step towards exploitation of aberrant PI-3-K signaling mechanisms in breast cancer prevention and chemotherapy.

HC11 MECs

HC11 and HC11-luci cells are widely used *in vitro* model systems for studying the episodic cycles of mammary gland epithelial cell growth, differentiation and apoptosis that occur throughout a female's life (Hynes, 1990; Cerrito, 2004; Petersen, 1998; Bailey, 2004). These cell lines were clonally derived from COMMA-1D cells obtained from mid-pregnant BALB/c mice (Ball, 1988; Hynes, 1990). The HC11 cell line is particularly useful in furthering the understanding of MEC differentiation; important elements of that process have been preserved in HC11 cells. HC11- luci cells possess the added feature of a luciferase reporter gene under the control of a β-casein promotor (Wartmann, 1996). Both of these cell lines display a normal phenotype, and injection of HC11 cells into the cleared fat pad of BALB/c mice exhibit normal ductal and alveolar-like structures (Humphreys, 1997).

HC11 MEC differentiation is initiated following growth to confluence in the presence of EGF. Removal of EGF followed by addition of DIP to confluent cells results in their differentiation as judged by milk protein production. Other universal markers of

MEC differentiation include cellular formation of acinar-like structures (mammospheres) and Jak2/Stat5A and B activation (Hynes, 1990; Xie, 2002; Petersen, 1998). HC11 and HC11-luci cells differentiate in response to lactogenic hormone exposure and express specific milk proteins, *e.g.,* β-casein and whey acidic protein (WAP), or, in the case of HC11-luci cells, β-casein promotor-driven luciferase expression. HC11 and HC11-luci cells induced to differentiate also form mammospheres and exhibit Jak2/Stat5A and B activation (Xie, 2002; Cerrito, 2004; Petersen, 1998). These cells also express receptor tyrosine kinases of various subclasses (Merlo, 1996; Hynes, 1994; Cerrito, 2004; Bailey, 2004), and mitogens (EGF, FGF) or oncogene products (activated Ras) inhibit lactogenic differentiation (Hynes, 1990; Merlo, 1996; Tonko-Geymayer, 2002; Cerrito, 2004). The similarity of these HC11 and HC11-luci cell responses to differentiation induction and those observed in normal peri-parturient female MECs emphasizes the utility of these cell lines in furthering our understanding of MEC differentiation and, ultimately, our understanding of the molecular pathogenesis of MEC-associated disease processes.

PART TWO

PAPERS

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Dominant Negative Ras Enhances Lactogenic Hormone-Induced Differentiation by Blocking Activation of the Raf–Mek–Erk Signal Transduction Pathway

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Epidermal growth factor (EGF) and Ras mitogenic signal transduction pathways are frequently activated in breast carcinoma and inhibit mammary differentiation and apoptosis. HC11 mouse mammary epithelial cells, which differentiate and synthesize β-casein following growth to confluency and stimulation with lactogenic hormones, were used to study EGF-dependent signaling during differentiation. Blocking Mek–Erk or phosphotidylinositol-3-kinase (PI-3 kinase) signaling with specific chemical inhibitors enhanced β-casein promotor-driven luciferase activity. Because EGF stimulation of HC11 cells resulted in the activation of Ras, the effect of activated Ras (RasV12) or dominant negative (DNRasN17) on lactogen induced differentiation was examined. HC11 cell lines expressing RasV12 or DNRasN17 under the control of a tetracycline (tet)-responsive promotor were constructed. Activated RasV12 expression resulted in reduced tyrosine phosphorylation of Stat5 and a delay in β-casein expression in response to prolactin. However, the expression of tet-regulated DNRasN17 and adenovirus-encoded DNRasN17 enhanced Stat5 tyrosine phosphorylation, Stat5 DNA binding, and β-casein transcription. The expression of DNRasN17 blocked the activation of the Mek–Erk pathway by EGF but did not prevent the phosphorylation of AKT, a measure of activation of the PI-3-kinase pathway. Moreover, the expression of DNRasN17 prevented the block to lactogenic differentiation induced by EGF. Stimulation of HC11 cells with prolactin resulted in the association of the SHP2 phosphatase with Stat5, and this association was prevented by DNRasN17 expression. These results demonstrate that in HC11 cells DNRas inhibits the Mek–Erk pathway and enhances lactogenic hormone-induced differentiation. This occurs, in part, by inhibiting the association of the SHP2 phosphatase with Stat5. J. Cell. Physiol. 201: 244–258, 2004. Published 2004 Wiley-Liss, Inc.†

Mammary epithelial cells undergo periodic cycles of growth, differentiation, and apoptosis during pregnancy and lactation. A complex series of signals that include mammotrophic hormones, locally derived growth factors and stroma initiate and regulate these processes. In this study, we address the problem of inhibition of mammary cell differentiation by mitogenic growth factors, including epidermal growth factor (EGF), that are found locally in the mammary gland. Because elevated levels of different growth factors in the EGF family such as transforming growth factor α (TGFα) and amphiregulin have been reported in breast tumors (Dotzlaw et al., 1990; Mizukami et al., 1991; Salomon et al., 1999) this study addresses an important issue in both normal development and neoplasia.

The HC11 mouse mammary epithelial cell line used in this study was derived from the COMMA-1D cell line, which was established from a midpregnant BALB/c

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mouse mammary gland (Danielson et al., 1984). This cell line has been employed as a model system for the study of regulation of mammary differentiation both in vitro and in vivo. HC11 cells introduced into the mammary fat pad differentiate into ductal-like structures (Humphreys and Rosen, 1997). In culture, HC11 mouse mammary epithelial cells differentiate and synthesize β-casein following growth to confluency and stimulation with the lactogenic hormone mixture, DIP (dexamethasone, insulin, prolactin) (Ball et al., 1988). β-casein expression in HC11 cells has been used as marker of differentiation, and its regulation in HC11 cells in culture reflects the in vivo regulation of expression of this protein in the mammary gland during pregnancy by prolactin (Ball et al., 1988; Peterson and Haldosen, 1998). Production of β-casein in cell culture is dependent upon both the presence of specific mitogens during the growth of the HC11 cells, cell–cell contact, deposition of extracellular matrix, and the subsequent prolactin-dependent activation of Stat5a and b when the cells have reached confluency (Taverna et al., 1991; Marte et al., 1995; Merlo et al., 1996). Prolactin stimulation results in Jak2-mediated tyrosine phosphorylation of Stat5a and b and nuclear translocation of these factors (Gouilleux et al., 1994; Marte et al., 1995; Han et al., 1997; Ali, 1998). In HC11 cells, the activation of Stat5 by prolactin is not dependent on the Ras–Erk pathway (Wartmann et al., 1996). However, the hormone-induced expression of β-casein can be blocked by the activation of different tyrosine kinase signaling pathways at the time of prolactin addition (Hynes et al., 1990; Marte et al., 1995; Merlo et al., 1996; Peterson and Haldosen, 1998). Previous studies have demonstrated that EGF prevents HC11 differentiation in response to lactogenic hormones. However, several signal transduction pathways have been implicated as responsible for the inhibition of β-casein synthesis. One study reported that the EGF-dependent inhibition of β-casein expression occurred through a Ras- and phosphoinositol-3′-kinase (PI-3 kinase)-dependent mechanism, not a Ras–Erk pathway (DeSantis et al., 1997; Salomon et al., 1999). More recently PTP-PEST, a phosphatase that can act on Jak2, was implicated as an EGF-induced protein contributing to this inhibition (Horsch et al., 2001).

Receptor tyrosine kinase (RTK) activation through different growth factor receptors leads to activation of Ras by guanine nucleotide exchange factors. The ErbB family of RTKs use this mechanism to stimulate signal transduction in the Ras pathway (Janes et al., 1994). Signal transduction that is downstream of Ras depends on the association of Ras GTPase with its effector proteins. Several proteins have been identified which associate with Ras in a GTP-dependent manner. These include Raf-1, RasGAP, p110 subunit of PI-3-kinase, AF6, Rin-1, Mek kinase 1, protein kinase C zeta, and RalGDS (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Kikuchi et al., 1994; Rodriguez-Vicania et al., 1994; Akasaka et al., 1996). Activation of Ras initiates a signaling cascade via activation of the Raf-1 and Mek-1 kinases resulting ultimately in the activation of Erk kinases (Shibuya et al., 1992; Moodie et al., 1993). The results of several studies have indicated that the activation of the Ras–Erk kinase pathway can either induce or enhance the differentiation of breast cancer cell lines (Bacus et al., 1992; Giani et al., 1998; Lessor et al., 1998). However, the activation of the Ras–Raf–Mek–Erk pathway by EGF inhibits hormone-induced differentiation in HC11 cells (Hynes et al., 1990), and the expression of v-Raf, which also activates Erk signaling, has a similar effect (Jehn et al., 1992; Happ et al., 1993).

In the present study, we have addressed the mechanism of EGF inhibition of HC11 lactogenic hormone-induced differentiation by examining the involvement of specific signal transduction pathways on differentiation. These studies indicated that the Ras–Mek–Erk pathway and, to a lessor degree, the PI-3 kinase pathway contribute to this inhibition by EGF. Moreover, the expression of DN Ras prevented the EGF-dependent disruption of HC11 differentiation indicating that Ras-signaling is central to this process. DN Ras expression blocked EGF-induced activation of the Mek–Erk signaling but not PI-3-kinase signal transduction, indicating that stimulation of the Mek–Erk pathway is the primary mechanism blocking lactogenic differentiation in HC11 cells.

MATERIALS AND METHODS

Cell culture

Mouse mammary epithelial cell lines, HC11 and HC11-luci, kindly provided by Dr. Nancy Hynes, were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml Insulin, 10 mM HEPES, and 10 ng/ml EGF as described (Hynes et al., 1990; Marte et al., 1995).

Lactogenic hormone-induced differentiation

The HC11 cells were grown to confluence and maintained for 3–5 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 5 μg/ml Insulin, 10 mM HEPES, 10 ng/ml EGF to establish competence (Ball et al., 1988; Taverna et al., 1991). To induce lactogenic hormone-induced differentiation EGF-containing media was removed, the cells were rinsed twice and then incubated in differentiation media, i.e., serum free- or serum containing-RPMI with dexamethasone (10^{-6} M), insulin (5 μg/ml), and prolactin (5 μg/ml) referred to as DIP. The cells were harvested at the stated times after the addition of DIP. Alternatively, HC11 Tet-off cell lines were grown to confluence for 6 days in EGF-containing media in the absence of doxycycline, then maintained for 24 h in media without EGF prior to the addition of DIP. HC11 differentiation was characterized in these cells by the formation of domed structures referred to as mammospheres (Blatchford et al., 1995; Humphreys and Rosen, 1997) which were enumerated by phase contrast microscopy. The cell cultures were photographed using 20× objective with a Nikon Ix70 camera.

Construction of cell lines

The HC11 cell line was transfected with pTetOff plasmid (BD Biosciences Clontech, Palo Alto, CA) using Lipofectamine 2000 (Introvitrogen Life Technologies, Carlsbad, CA). The cells were incubated in G418 (200–500 μg/ml) selection media for 10 days, individual colonies were picked with cloning cylinders and expanded in 24-well plates. The colonies were screened

for the regulation of the Tet-promotor by transfection with a Tet-promoter-luciferase construct and incubation in medium with and without doxycycline (0–0.5–2.0 μg/ml). The promotor activity was assessed using a luciferase assay system (Promega, Madison, WI) with the light emission measured in a luminometer and expressed as light intensity/μg cell protein. Two cell lines exhibited up to 40-fold increase in a Tet-responsive promotor in response to the removal of doxycycline from the cultures. These HC11 tet-off cell lines were used for the construction of cell lines expressing specific genes under the control of the Tet-responsive element (TRE).

The HC11 Tet-off cell lines were infected with retroviral vectors expressing Tet-regulated *Ki-Ras* genes. pREV-TRE (Clontech), a retroviral vector that expresses a gene of interest from TRE, was derived from pLNCX, a Moloney murine leukemia virus (MoMuLV)-derived retroviral vector. The TRE contains seven direct repeats of the 42-bp tetO operator sequence, which can be bound by tTA transactivators, upstream of a minimal CMV promoter. The 5′ viral LTR regulates expression of the transcript that contains the viral packaging signal and the hygromycin resistance (*Hyg*^{r}) gene. The TRE is an internal promotor in this vector. pREV-TRE was used to inducibly express the Ki-Ras genes in response to removal of doxycycline (Dox).

pREV-TRE-RasV12 (active K-Ras 2B-V12) and pREV-TRE-DNRasN17 (dominant negative K-Ras 2B-N17) plasmids were constructed by introduction of K-Ras cDNA into pREV-TRE plasmid and selection on hygromycin. For the production of retroviral vector stocks 1.5×10^5 PA317 packaging cells were transfected with 1 μg of recombinant retroviral vector DNA and Lipofectamine 2000 in a 35 mm well. Twenty-four hours post-transfection the PA317 cells were split and selected in hygromycin containing media (100 μg/ml) for 10 days. Mass cultures were prepared from approximately 50–100 colonies and used to produce retroviral vectors stocks. At this point, viral titers were high enough to use for retroviral infection of HC11 Tet-off cells. The HC11-Tet-off cell line was infected with pREV-TRE, pREV-TRE-RasV12, and pREV-TRE-DNRasN17 vector stocks. Cells were selected in hygromycin (100 μg/ml) and doxycycline (2 μg/ml) for 10 days. Six colonies from each HC11 Tet-off infected cell line were isolated and expanded into cell lines. The clonal cell lines were tested for expression of vector encoded Ras RNA by Northern blot following the removal of doxycycline.

Adenovirus infection

HC11 and HC11-luci cells were infected with replication defective adenoviruses. A control vector encoding on β-galactosidase (pAd-CMV-β-gal) or a vector encoding Ha-Ras N17, kindly provided by Dr. Craig Logsdon, were used for these experiments (Nicke et al., 1999). Cells were infected with 10–50 MOI of cesium chloride gradient-purified adenovirus by incubation of cells in a low volume of virus-containing media for 5–6 h. The virus was removed and media was added to the cells for 24 h prior to additional treatment of the cells.

Luciferase assays

HC11 luci cells were induced to differentiate in DIP-induction media with and without EGF (10 ng/ml). Inhibitors were added at the time of DIP-induction. Inhibitors were added at optimal concentrations (PD98059, 20 μM; LY294002, 10 μM; wortmannin, 100 nM) determined by dose–response curves (data not shown). Cell lysates were harvested 48 h after transfer to DIP-induction media and luciferase activity was determined using a commercial kit (Luciferase Assay System, Promega) and a luminometer (Thermolab Systems, Ascent, FL). The total cell protein was determined by BCA assay (Pierce, Rockford, IL) and luciferase activity was normalized to protein for all the samples. Results are presented as relative units calculated from the mean of six determinations.

Electrophoretic mobility shift assay (EMSA)

HC11 cells were grown to confluency in media containing 10% fetal calf serum, 10 ng/ml EGF, and 5 μg/ml insulin then maintained for 3 days without EGF. The cells were then starved for 24 h in serum-free media prior to induction for 15 min with DIP as described above. Nuclear extracts were prepared according to a previously published protocol with little modification (Wartmann et al., 1996). Briefly, harvested cells were suspended in CEB (10 mM KCl, 20 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 0.1% Triton X-100, 20% glycerol, 0.1 mM EGTA, 0.5 mM dithiothreitol, 2 mM sodium orthovanadate, 50 μM β-glycerophosphate, 50 mM sodium fluoride, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin) and sheared with 20 strokes using a Dounce homogenizer (Wheaton, pestle B). Nuclei were pelleted by centrifugation at 800*g* for 5 min and then extracted with NEB (CEB + 300 mM NaCl) by incubating for 30 min on ice. Extracts were clarified by centrifugation for 5 min at 16,000*g*. EMSAs were performed by incubating 10 μg of nuclear protein with the Stat5 DNA binding site from the bovine β-casein promotor (5′-AGATTTCTAGGAATTCAATCC-3′) or Sp1-binding oligonucleotide, end-labeled with ^{32}P-γ-ATP, for 30 min on ice in 16 μl of EMSA buffer (10 mM HEPES, pH 7.6, 2 mM NaH_2PO_4, 0.25 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 80 mM KCl, 2% glycerol, and 100 μg/ml poly [dI-dC]). Specific binding was analyzed on 6% DNA retardation gel and pre-run for 2 h at 200 V in 0.25 × TBE (22.5 mM Tris borate, pH 8.0, 0.5 mM EDTA) at 4°C. The samples were loaded and electrophoresed for 2 h at 200 V, the gels were dried and autoradiographed. For antibody supershift assays, nuclear extracts were pre-incubated with Stat5b C17 antibody (Santa Cruz) for 20 min prior to the addition of the labeled probe.

Northern blots

Total RNA was extracted using TriPure reagent (Roche). Northern blots were prepared using 7.5 or 10 μg of total RNA separated on 1% agarose-formaldehyde gel and transferred to a nylon filter. Blots were hybridized as described previously (Masuelli et al., 1999). The probes used included: mouse β-casein, human KiRas2B, and mouse actin. Mouse β-casein probe is a 601 bp fragment (nucleotide 3–603) from the mouse β-casein cDNA, (accession number X04490.1); it was obtained by RT-PCR and TA-cloning into PCR2.1 and sequence verified. The Ki-Ras probe is a 650 bp fragment

representing the human Ki-Ras 2b cDNA, and the actin probe was obtained from Clontech. Mouse Socs-3 probe consisted of nucleotides 467-1006 (accession number NM_007707.2) and mouse Cis-1 was nucleotides 526-1046 (accession number NM_009895.2).

MTT assay

The rate of replication of HC11-TRE and HC11-DNRasN17(12) cell lines was determined by proliferation assay using MTT dye (CellTiter96 Assay by Promega). The cells were propagated for 96 h in the absence of doxycycline. The viable cells were counted by 0.4% trypan blue dye exclusion test and the cell count was adjusted of 1×10^6 cells/ml in RPMI with 0.5% FBS. Cells were plated at density of 1.5×10^3 per well in quadruplicate wells in 96-well plate with or without EGF (10 ng/ml) incubated at 37°C for 24, 48, or 72 h. For analysis of proliferation 15 μl of MTT dye solution was added to each well and the culture plate was incubate at 37°C in CO_2 incubator for 4 h. After 4 h 100 μl of solubilization-stop solution was added to each well. Following 1-h incubation at 37° C the samples were mixed by pipeting and the optical density was measured at 570 nm. The mean and standard deviation of the absorbance values for the quadruplicate wells were calculated.

Immunoprecipitations and Western blots

HC11 cell lysates were prepared in triton-glycerol buffer (1% Triton-X 100, 10% glycerol, 25 mM HEPES, 150 mM NaCl, 2 mM EDTA), NP40 buffer (1% NP40, 25 mM HEPES, 150 mM NaCl) or high salt buffer (Wyszomierski et al., 1999). All lysis buffers contained AEBSF (20 μg/ml), aprotinin, (5 μg/ml), leupeptin, (5 μg/ml), β-glycerol phosphate (100 μM), and $NaVaO_4$ (1 mM). Immunoprecipitates were prepared by incubation of 0.5 or 1 μg of primary antibody with equal amounts of protein (400 μg) and collected by binding to Protein A agarose (Invitrogen Life Technologies, Carlsbad, CA). Antibodies include anti-Stat5, sc-835 (SantaCruz Biotechnology, Santa Cruz, CA), anti-phosphoStat5 (Cell Signaling Technology, Beverly, MA). For Western blots equal amount of protein were separated by SDS–PAGE and transferred to PVDF filters. Filters were blocked with 2.5% nonfat dried milk (Blotto) in TBS-T for 1 h, then incubated with the appropriate dilution of antibody for 1 h at room temperature or 16 h at 4°C with agitation. Following the incubation with HRP-labeled secondary antibodies, blots were washed and reactivity was detected using ECL (Amersham). Blots were either exposed to Kodak XAR film or results were quantified using a CCD camera (Fuji). Films were quantitated by densitometry. Antibodies included anti-Stat5, sc-835 (SantaCruz), anti-phosphoStat5 (Cell Signaling), anti-phospho Erk, V8031 (Promega), anti-pan Erk (Transduction), anti-AKT and anti-phosphoAKT-ser 473 (Cell Signaling), anti-SHP2 (Transduction), anti-Mek1,2 (Transduction). Anti-PTP-PEST was supplied by Dr. Michael Schaller. Antibodies purchased from Santa Cruz Biotechnology were used at 1 μg/ml, and the antibodies from other suppliers were used at the dilution suggested by the manufacturer.

RESULTS

EGF blocks hormone-induced HC11 differentiation through Mek and PI-3-kinase-dependent pathways

Previous studies demonstrated that EGF blocked lactogenic hormone-induced differentiation of HC11 cells (Hynes et al., 1990), and recent data suggested that this block required Ras and PI-3-kinase activity (DeSantis et al., 1997). In the present study specific chemical inhibitors of signal transduction pathways were used to further analyze the contribution of individual signaling pathways to the block of HC11 differentiation by EGF. Because lactogenic hormone-induced differentiation of HC11 cells is characterized by the initiation of β-casein transcription, the HC11-luci cell line, which contains a β-casein promotor linked to the luciferase gene, was used to provide a rapid readout of the differentiation process.

The HC11-luci cells were induced to differentiate with DIP in the absence and presence of EGF. Specific inhibitors of Mek, and PI-3-kinase were added to cells at the time of induction of differentiation. As expected there was a significant inhibition of β-casein driven luciferase activity in the EGF-treated samples compared to the DIP control. However, several compounds (PD98059, LY294002, and wortmannin) restored the β-casein promotor driven luciferase activity that was blocked by EGF (Fig. 1A). The results demonstrated that the inhibition of Mek–Erk signaling by PD98059 and PI-3-kinase signaling by LY294002 and wortmannin disrupted the EGF signaling that inhibited lactogenic hormone-induced differentiation, as measured by the activation of β-casein promotor driven luciferase expression.

The effect of chemical inhibitors of signal transduction pathways on the synthesis of β-casein RNA was examined (Fig. 1B). The results confirmed that exposure of HC11 cells to DIP activated β-casein expression and that EGF reduced the expression. The inclusion of PI-3-kinase or Mek1 inhibitors in the induction media with EGF reversed the EGF-induced inhibition of the endogenous β-casein promotor activity in the HC11-luci cells.

Previous studies demonstrated that the treatment of HC11 cells with DIP resulted in increased Stat5 DNA binding and that the DNA binding activity of Stat5 was reduced by the simultaneous addition of EGF and lactogenic hormones (Marte et al., 1995). Therefore, EMSA was performed to examine the ability of the signal transduction inhibitors to alter Stat5 DNA binding. Nuclear extracts were prepared from HC11 cells induced to differentiate in the presence of Jak2, Mek1, or PI-3-kinase inhibitors. The results of this reproducible experiment indicated that DIP stimulation in the presence of the Mek1 (PD98059) and PI-3-kinase (wortmannin) inhibitors enhanced Stat5 binding to DNA compared to the binding detected with DIP alone (Fig. 2A). In contrast, exposure of the HC11 cells to DIP plus AG490, an inhibitor of Jak2 tyrosine phosphorylation, inhibited Stat5 DNA binding (Fig. 2A, lanes 4 and 8). The results in Figure 1 indicated that Mek1 and PI-3-kinase inhibitors restored the DIP-induced Stat5 promotor activity inhibited by EGF, and the same Mek and

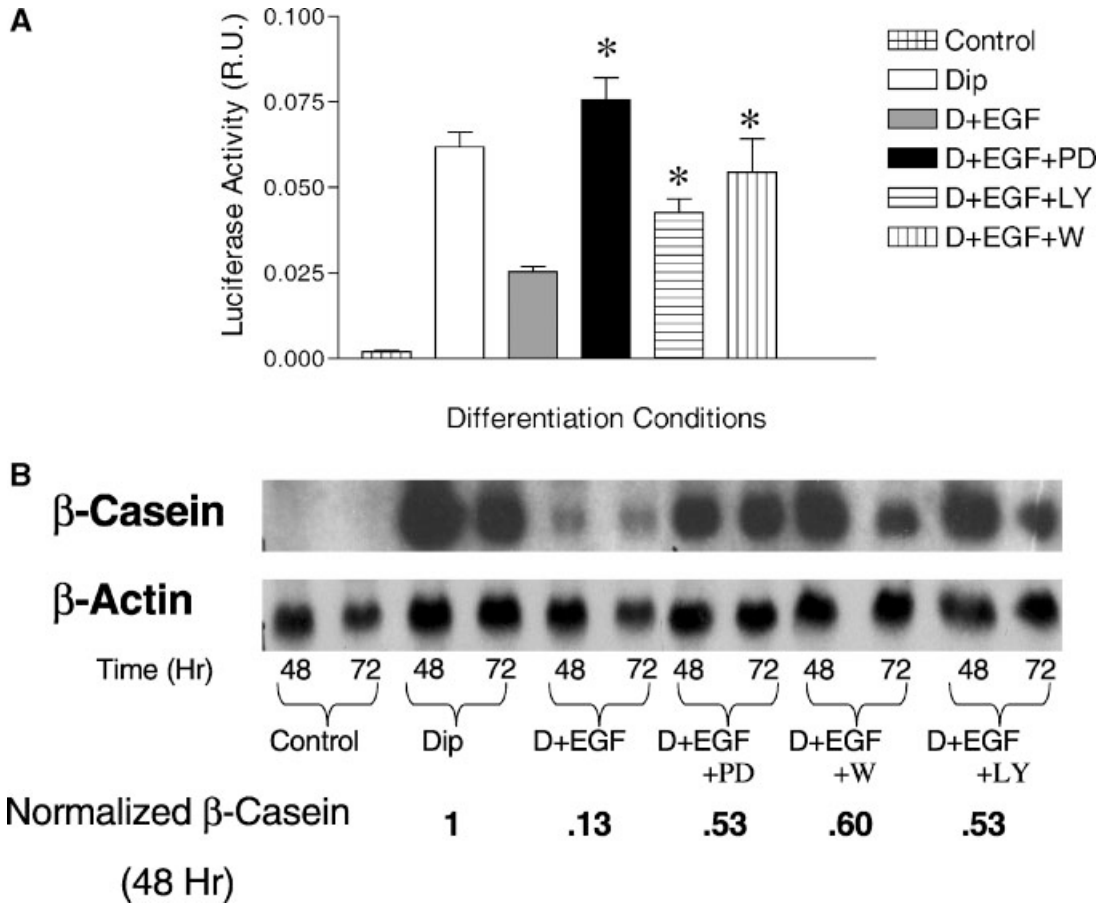


Fig. 1. **A**: The effect of signal transduction inhibitors on epidermal growth factor (EGF) disruption of differentiation. HC11-luci cells were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media with serum in the presence or the absence of EGF (10 ng/ml). Inhibitors were added at the time of DIP induction at previously determined optimal concentrations (PD98059, 20 μM; LY294002, 10 μM; wortmannin, 100 nM). The luciferase activity in lysates was determined at 48 h post-induction. Luciferase activity was normalized to cell protein. The results, presented as luciferase activity in relative units and represent the mean of six determinations. *, These values represent statistically significant difference (P value 0.001) from the DIP + EGF condition. **B**: The effect of signal transduction inhibitors on EGF disruption of β-casein transcription in HC11 cells. The HC11 cells were induced to differentiate in DIP-induction media with and without EGF (10 ng/ml). Inhibitors were added at the time of induction at slightly lower than optimal concentrations to avoid toxicity (PD98059, 10 μM; LY294002, 5 μM; wortmannin, 50 nM). Total cell RNA was harvested at 48 or 72 h after transfer to DIP-induction media. β-Casein induction was determined via Northern blot. For quantitation β-casein expression at 48 h was normalized to β-actin. The level of expression in DIP-treated cells was set as 1.

PI-3-kinase inhibitors enhanced Stat5 DNA binding. Blocking the Mek–Erk and PI-3-kinase pathways with specific inhibitors both enhanced HC11 markers of differentiation and prevented the EGF-dependent disruption of HC11 differentiation.

HC11 cells expressing dominant negative RasN17 exhibit an enhanced lactogenic differentiation response

Because Ras activation regulates the activation of the Erk pathway by EGF and may contribute to the activation of PI-3-kinase, the role of Ras activation in the disruption of HC11 differentiation by EGF was examined. HC11 cell clones expressing either activated Ki-Ras (RasV12) or dominant negative Ki-Ras (DNRasN17) were constructed as described in Materials and Methods. The HC11 cell lines constructed contained the Ras cDNAs under the control of a Tet-responsive promotor in a Tet-off system. Hence, the expression of Ras increased following the removal of doxycycline from the culture media. Several independent clones containing each vector were isolated and characterized for the inducibility of Ras gene expression following the removal of doxycycline from the cultures. As expected, the inducibility varied for the individual RasV12 and DNRasN17 clones. The results obtained with three independent clones derived from each vector are shown in Figure 3.

The DNRasN17 and the RasV12 HC11 cell lines were compared to the vector control cell line, REV-TRE, to determine the effect of the Ras gene expression on lactogenic hormone-induced differentiation. HC11 transfectant cell lines expressing DNRasN17 or activated RasV12 along with the vector control cell line were grown for 72 h in the absence of doxycycline at which point the confluent cultures were incubated in DIP differentiation media. RNA was harvested from cells at 0, 48, 72, and 96 h post-addition of DIP and used to

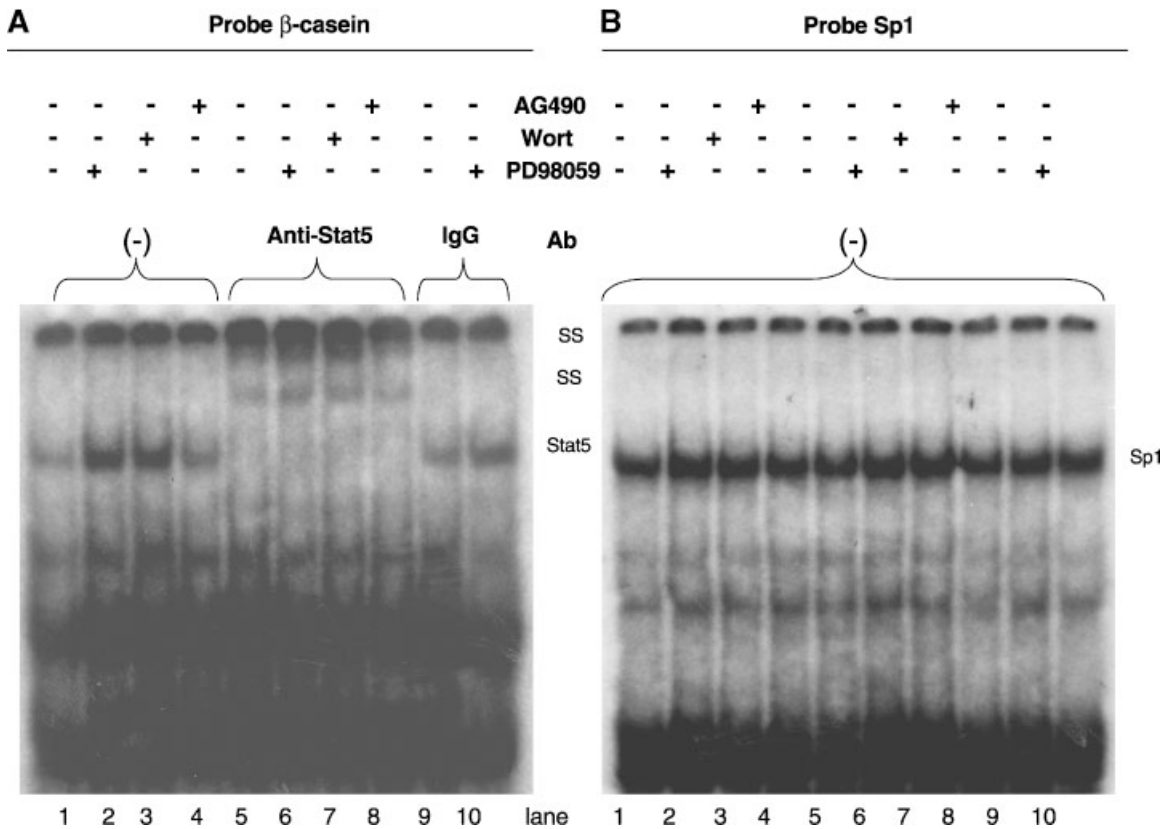


Fig. 2. The effect of inhibitors on Stat5 DNA binding by EMSA. **A**: HC11 cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for 3 days and serum-free media for 1 day. HC11 cells were pretreated with specific kinase inhibitors for 2 h prior to DIP-induced differentiation for 15 min in the presence of the inhibitors. Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody. Lanes 1 and 5: Control (DIP alone); (lanes 2 and 6) PD 98059 (20 μM) plus DIP; (lanes 3 and 7) wortmannin (20 nM) plus DIP; (lanes 4 and 8) AG 490 (20 μM) plus DIP. Lanes 5–8: The binding was performed in the presence of anti-Stat5 antibody for supershift. Lane 9, 10: The samples were the same as (lanes 1, 2) but rabbit IgG was added. **B**: Gel shift (control) using Sp1 oligos as a loading control. The same protein lysates were used as in Part A, but the binding was to an Sp1 oligonucleotide. SS, supershift of Stat5.

determine the level of Ras and β casein expression by Northern blotting. The results in Figure 3 indicated that RasV12 expression inhibited β-casein expression by approximately 50% compared to the TRE control cell line. In contrast, the expression of DNRasN17 enhanced β-casein induction up to twofold compared to the control. The results demonstrated that the amount of DNRasN17 expression correlated with the effect on differentiation. The HC11 cell clone expressing the greatest amount of DNRasN17 (clone 12) exhibited the greatest level of β-casein expression. In contrast, all clones of expressing RasV12 inhibited β-casein expression.

The effect of Ras expression on mammosphere formation, a phenotypic measure of differentiation for primary mammary epithelial cells as well as HC11 cells, was determined. Following growth in the absence of doxycycline, EGF was removed from the cells and lactogenic differentiation was induced by the addition of DIP. The cells were photographed at 0, 72, and 120 h post-DIP and the number of domed mammospheres that appeared in each culture were enumerated (Fig. 4A). At 72 h after DIP addition, the mammospheres were easily counted, but by 120 h the size and the number in the DNRas cell line were too great to count. The results indicated that mammosphere formation was inhibited by RasV12 expression and was significantly enhanced by DNRas expression.

In parallel experiments, the effect of Ras expression on the prolactin-induced tyrosine phosphorylation of Stat5 was examined. HC11 TRE vector control cells as well as the RasV12 (clone 1) and DNRasN17 (clone 12) cells were stimulated with DIP, and the phosphorylation status of the Stat5 protein was determined by immunoprecipitation and Western blotting using anti-Stat5 tyrosine 694 (Y694) phosphorylation site-specific antibodies. The results, seen in Figure 4B, indicated that the tyrosine phosphorylation of Stat5 was enhanced and sustained in the DNRasN17 HC11 cell line compared to the TRE vector control cell line. However, the tyrosine 694 phosphorylation was of a shorter duration in the cell lines expressing activated RasV12 than in the TRE control cells. These results suggested that Ras-dependent signal transduction can modulate Stat5 phosphorylation in HC11 cells in response to DIP. The Stat5 EMSA results supported this conclusion (Fig. 4C). Enhanced Stat5 DNA binding in response to DIP stimulation was observed in the DNRasN17 HC11 cell

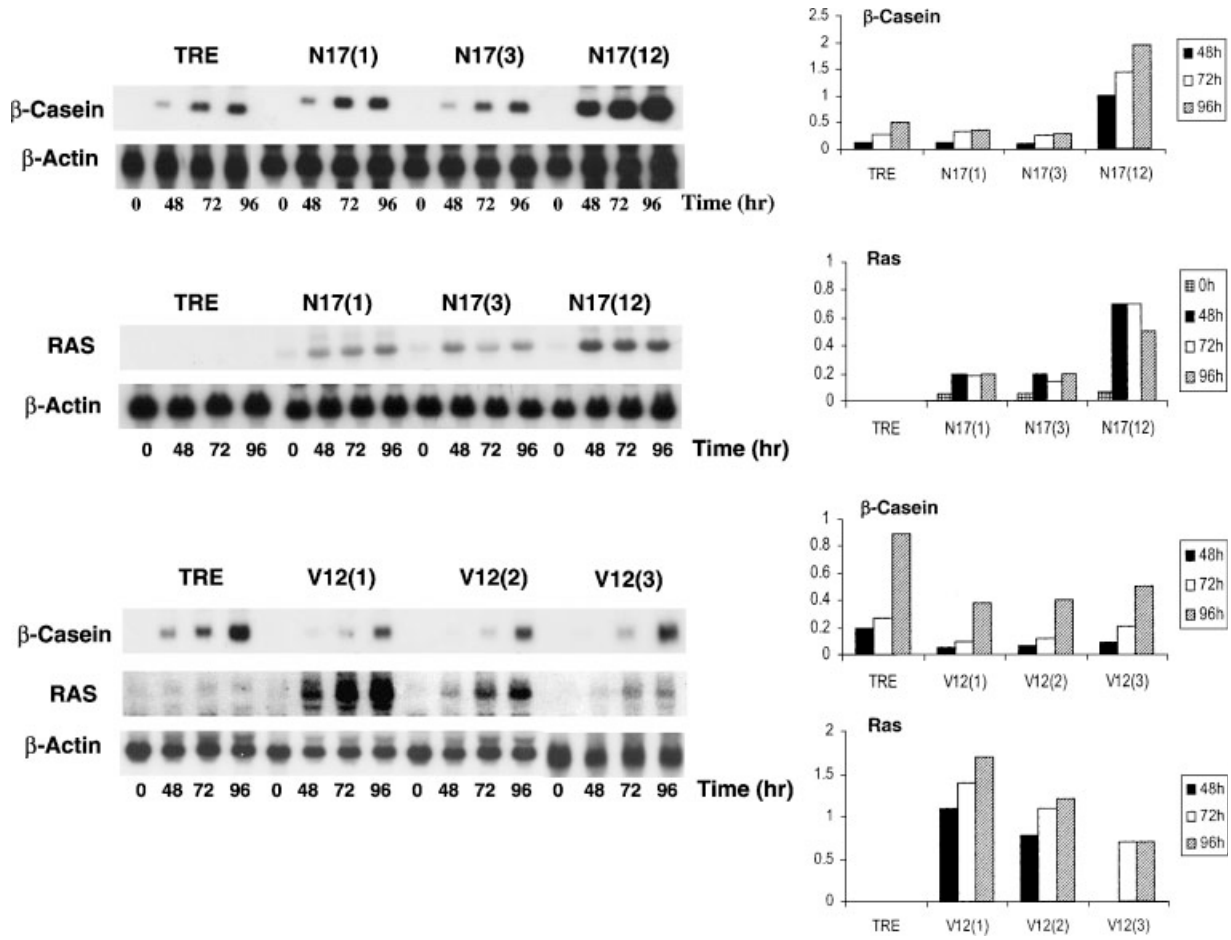
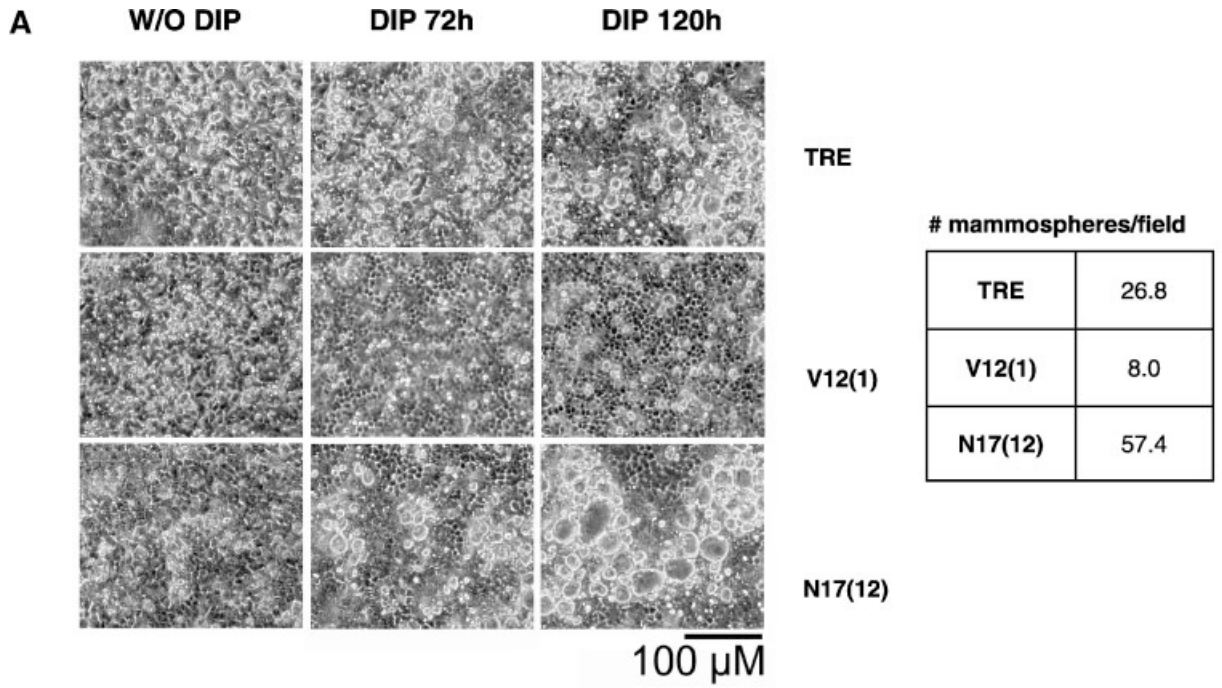


Fig. 3. The effect of RasV12 and DNRasN17 on lactogenic differentiation. HC11 cells expressing activated RasV12 and DNRasN17 under the control of the Tet-responsive promotor were utilized to evaluate the effect of Ras-based signal transduction on lactogenic differentiation. Three individual clones of HC11 cells expressing either RasV12 (clones 1–3) or RasN17 (clones 1, 3, 12) under the control of the Tet responsive promotor were grown to confluence, incubated in the absence of doxycycline and exposed to DIP differentiation media. The vector control cell line, TRE, was treated in parallel. RNA was harvested from cells at 0, 48, 72, and 96 h after addition of DIP and used to determine the level of Ras and β-casein expression by Northern blotting. The Ras and β-casein expression was quantitated using a beta scanner and were normalized to the actin signal and reported in relative units.

lysates as compared to the vector control. In contrast, the Stat5 DNA binding activity was reduced in cells expressing activated RasV12. In conclusion, an increase in HC11 cell lactogenic hormone-induced differentiation is observed following blockade of the Ras signaling pathway. Moreover, in the HC11 cells that have Ras activity blocked, the enhancement of hormone-induced differentiation appeared to be attributable to an increase in Stat5 tyrosine phosphorylation and to an increase in Stat5 DNA binding resulting in enhanced transcription of β-casein, a Stat5-regulated gene.

Fig. 4. The effect of RasV12 and DNRasN17 expression on mammosphere formation, Stat5 phosphorylation and DNA binding. **A**: HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12 (clone 1) or DNRasN17 (clone 12) were grown to confluence and exposed to DIP as described in Materials and Methods. The cells were photographed at 0, 72, and 120 h post-DIP addition. The number of mammospheres per field is reported; this was determined by counting the number of mammospheres per low power field and determining the mean of five fields. **B**: HC11 TRE vector control cells and HC11 cell lines expressing activated RasV12 (clone 1) or DNRasN17 (clone 12) were grown to confluence in EGF-containing media without doxycycline to induce the expression of Ras. The cells were stimulated with DIP, and nuclear extracts were prepared from cells at 0, 15 min, 1 and 24 h post-stimulation. Total Stat5 was immuno-precipitated and analyzed by Western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 on the Western blots was quantitated, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. **C**: EMSA. HC11 TRE vector control, RasV12(1), and RasN17(12) cells were grown to confluence in EGF-containing media, then incubated in EGF-free media for 3 days and serum-free media for 1 day. Treated cells were exposed to differentiation media for 15 min and control cells (T = 0) were not exposed to DIP. Left part: Nuclear lysates were prepared and used for Stat5 binding to the β-casein GAS element in the presence or absence of anti-Stat5 antibody as indicated. Lanes 1, 4, 7: TRE control; (lanes 2, 5, 8) RasV12 (1); (lanes 3, 6, 9), RasN17(12). Right part: Sp1 binding oligonucleotides were used as a loading control. Lanes 1, 4, 7: TRE control; (lanes 2, 5, 8) RasV12(1); (lanes 3, 6, 9) RasN17(12). Lanes 1–3: Contain control lysate; (lanes 4–6) contain lysate from DIP-treated cells; (lanes 7–9) contain lysate from DIP-treated cells with the addition of 50 × cold Sp1 oligonucleotides. SS: Stat5 supershift.


A
W/O DIP
DIP 72h
DIP 120h
TRE
V12(1)
N17(12)
100 μM
mammospheres/field
TRE 26.8
V12(1) 8.0
N17(12) 57.4
B
P-STAT5
STAT5
0' 15' 1hr 24hr
0' 15' 1hr 24hr
0' 15' 1hr 24hr
TRE
V12(1)
N17(12)
Stat5 Phosphorylation (R.U.)
1.5
1.0
0.5
0.0
15'
1hr
24hr
TRE
V12(1)
N17(12)
C
DIP 0 min 15 min 15 min+Ab
C V12 N17 C V12 N17 C V12 N17
SS
SS
Stat5
lane
1 2 3 4 5 6 7 8 9
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C V12 N17 C V12 N17 C V12 N17
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Fig. 4.

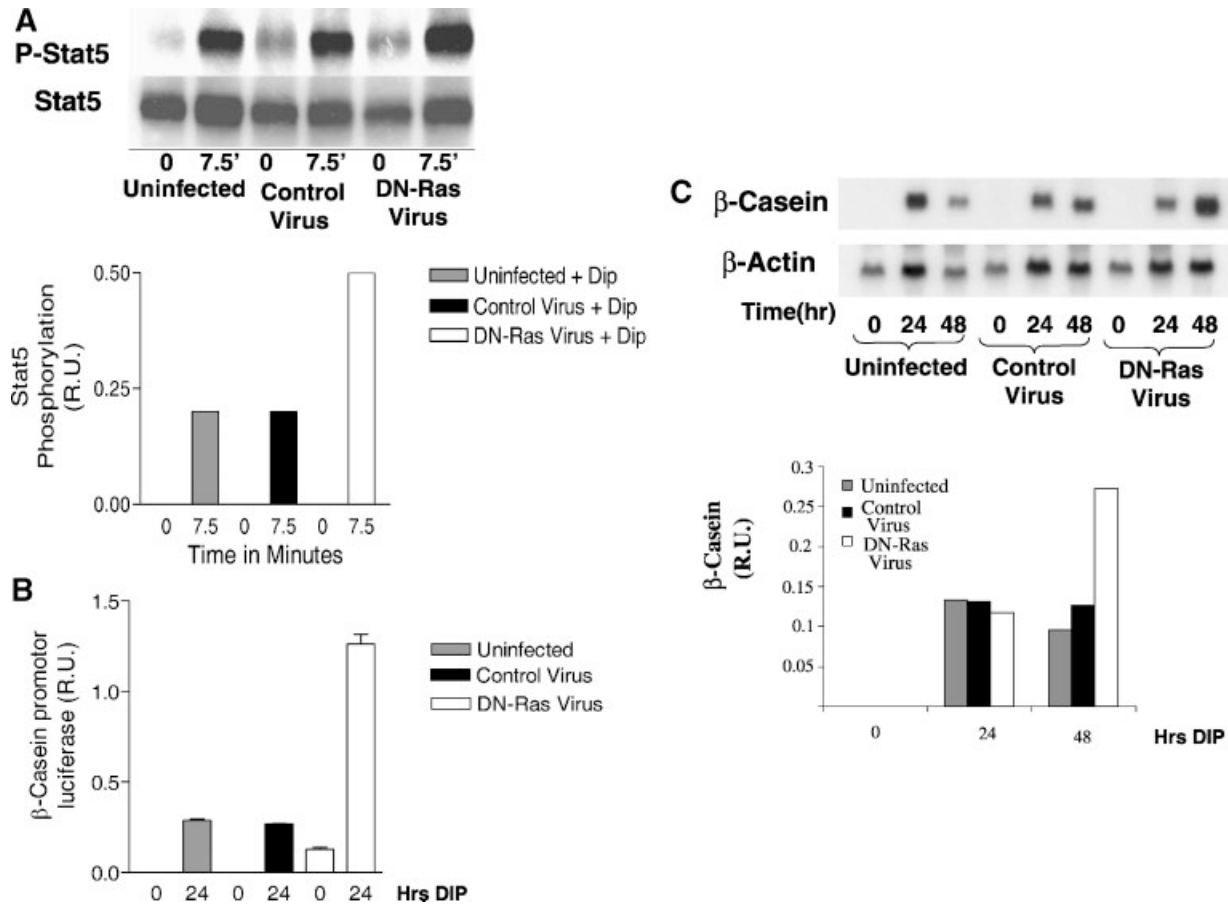


Fig. 5. The effect of dominant negative Ha-Ras (N17) adenovirus expression on lactogenic differentiation in HC11 cells. **A**: The effect of DNRasN17 adenovirus on Stat5 phosphorylation in response to lactogenic hormone was determined. Uninfected HC11 cells, HC11 cells infected with a control adenovirus vector and HC11 cells infected with adenovirus encoding DNRas (N17) (at MOI = 10) were incubated for 24 h; the cells were then serum-starved overnight and stimulated with DIP for 7.5 min. Total Stat5 was immunoprecipitated and analyzed by Western blotting with antibodies for phospho-Stat5 or total Stat5. The amount of phospho-Stat5 and total Stat5 was quantitated using a CCD camera, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units. **B**: HC11-luci cells infected with adenovirus vector control or adenovirus encoding dominant negative RasN17 were used to determine the effect of DNRasN17 on β-casein driven luciferase activity. The cells were infected with the viruses described above and incubated for a period of 24 h in media without EGF. The cells were then either stimulated with DIP for 24 h or incubated in media without EGF for an additional 24 h. The luciferase activity in lysates was determined and normalized to cell protein; the results, presented as luciferase activity in relative units, represent the mean of four determinations. **C**: The effect of DNRasN17 adenovirus infection on HC11 expression of β-casein was determined. The HC11 cells were infected with the control or DNRasN17 virus as described above. RNA was isolated at 0, 24, and 48 h post-induction of differentiation and used to determine the amount of β-casein transcription by Northern blotting. Hybridization of the blots with an actin probe was used as a control for RNA loading. The expression of the β-casein RNA was quantitated by measurement on a β-scanner, normalized to actin and expressed on a relative scale.

Infection of HC11 cells with dominant negative Ha-Ras adenovirus enhances lactogenic differentiation

Infection of cells with replication defective adenovirus encoding dominant negative Ha-RasN17 (DNRasN17) was used as another mechanism to examine the influence of the Ras pathway on lactogenic differentiation. HC11 cells and HC11-luci cells were infected with 10 MOI of either replication defective control adenovirus or adenovirus encoding DNRasN17. At 48 h post-infection, the cells were examined for the effect of DNRasN17 on Stat5 phosphorylation, β-casein promotor activity and β-casein RNA levels. As demonstrated in Figure 5A HC11-luci cells infected with control virus or DNRasN17 virus were stimulated with DIP and the level of Stat5 tyrosine 694 phosphorylation was determined. The results indicated that the expression of DNRasN17 increased the level of Stat5 phosphorylation in response to DIP compared to either uninfected or vector control-infected cells. HC11-luci cells infected with either replication defective control adenovirus or adenovirus encoding DNRasN17 were tested for activation of β-casein promotor-driven luciferase activity (Fig. 5B). There was a fivefold increase in the activation of luciferase activity in the DNRasN17 cells compared to the uninfected cells or the control adenovirus infected cells. In addition, there was some activation of luciferase activity in cells infected with the DNRasN17 virus without DIP exposure. This result was reproducible and is not seen when uninfected cells or vector infected cells were exposed to DIP. Finally, HC11 cells infected with

either replication defective control adenovirus or adenovirus encoding DNRasN17 were examined for expression of the endogenous β-casein gene following exposure to DIP for 24 or 48 h. The results of Northern blots (Fig. 5C) indicated that the infection with DNRasN17 virus resulted in a twofold increase in β-casein RNA compared to the uninfected or vector infected cells exposed to DIP.

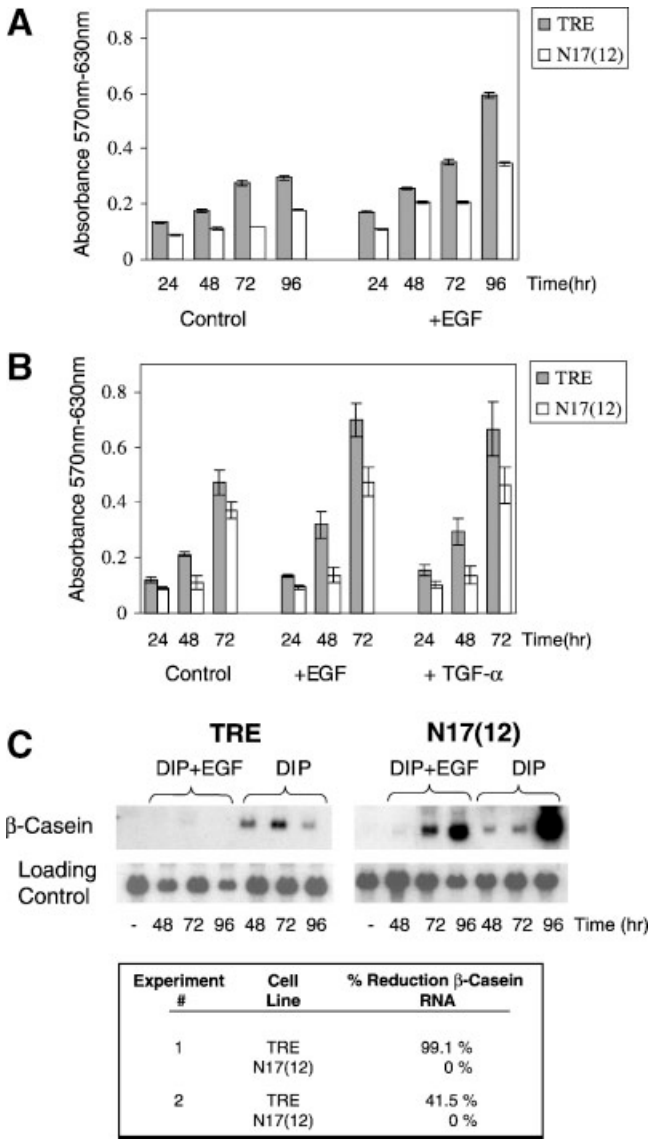
HC11 cells expressing dominant negative Ras exhibit reduced response to EGF

Studies were performed to determine if the DNRasN17 expression could block EGF-induced responses in stable transfectants of HC11 cells. HC11 cells respond mitogenically to EGF. The TRE vector control cells and the DNRasN17 cells were stimulated with EGF and the ability of the cells to proliferate was examined using the MTT assay. Cells were removed from doxycylcine for 96 h and then grown in reduced serum media in the absence and the presence of EGF. MTT assays were performed over the course of 4 days to follow cell proliferation. The results in Figure 6A demonstrated that the DNRasN17 cell line was growth inhibited by 40% in both the absence and presence of EGF compared to the vector control cell line. This experiment was repeated using TGFα treatment of HC11 vector control and DNRasN17 cells. Again, the DNRasN17 cells exhibited a significantly lower response to EGF and TGFα than did the vector control cell line (Fig. 6B).

The ability of DNRas to prevent the disruption of lactogenic hormone-induced differentiation by EGF in HC11 cells was examined. The HC11 TRE vector control cells and cells expressing DNRasN17 under the control of a Tet-responsive promotor were grown in the absence of doxycycline for 72 h. The cells were exposed to lactogenic hormone differentiation media in the presence and absence of EGF for varying lengths of time; RNA was extracted and the level of β-casein mRNA was analyzed by Northern blotting. The results in Figure 6C demonstrated that EGF did not inhibit the induction of β-casein transcription in the DNRasN17 cell line and, hence, it appeared that differentiation proceeded in these cells even in the presence of EGF. In contrast, the expression of β-casein was blocked by EGF in the TRE vector control cell line in two separate experiments. These results demonstrated that DNRasN17 expression prevented the disruption of hormone-induced differentiation by EGF in HC11 cells.

HC11 cells expressing dominant negative Ras exhibit reduced Erk activation in response to EGF

HC11 cells expressing DNRasN17 were examined to determine if expression of DN Ras prevented the activation of Mek–Erk or PI-3-kinase signaling in response to EGF. In Figure 7A the stable transfectants were removed from doxycycline and grown to confluence. The cells were starved and then stimulated with EGF for varying amounts of time. Cell lysates were prepared and analyzed by Western blot using antibodies that detect phosphorylated forms of different signaling proteins. The results, shown in Figure 7A, revealed that stimulation of HC11 vector control cells with EGF resulted in activation of p44Erk as detected by reactivity



Experiment #	Cell Line	% Reduction β-Casein RNA
1	TRE	99.1 %
	N17(12)	0 %
2	TRE	41.5 %
	N17(12)	0 %

Fig. 6. DNRas N17 expression inhibits EGF-induced proliferation and prevents EGF-dependent disruption of lactogenic differentiation. **A**: HC11 TRE vector control and DNRasN17 (clone 12) cells were grown in absence of doxycycline and then seeded in microtiter plates in 0.5% serum-containing media with and without EGF (10 μg/ml). Cell proliferation was determined at 24, 48, 72, 96 h post-addition of EGF using the MTT assay. The results are reported as the mean of four determinations. **B**: The HC11 TRE vector control and DNRasN17 (clone 12) cells were grown as described above and exposed to EGF (10 ng/ml) or TGFα (10 ng/ml). Cell proliferation was determined using the MTT assay and the results represent the mean of four determinations. **C**: HC11 TRE vector control and RasN17 (12) cells were grown to confluence in absence of doxycycline and then exposed to DIP in the presence or absence of EGF (10 ng/ml). Total RNA was isolated after 72 h and used for Northern blotting. The blots were hybridized to probes for β-casein and actin. The β-casein and actin RNA was quantitated using a beta scanner; the β-casein RNA was normalized to the actin RNA. The % reduction of β-casein RNA by the addition of EGF during DIP-induced differentiation was calculated using the values for normalized β-casein expression.

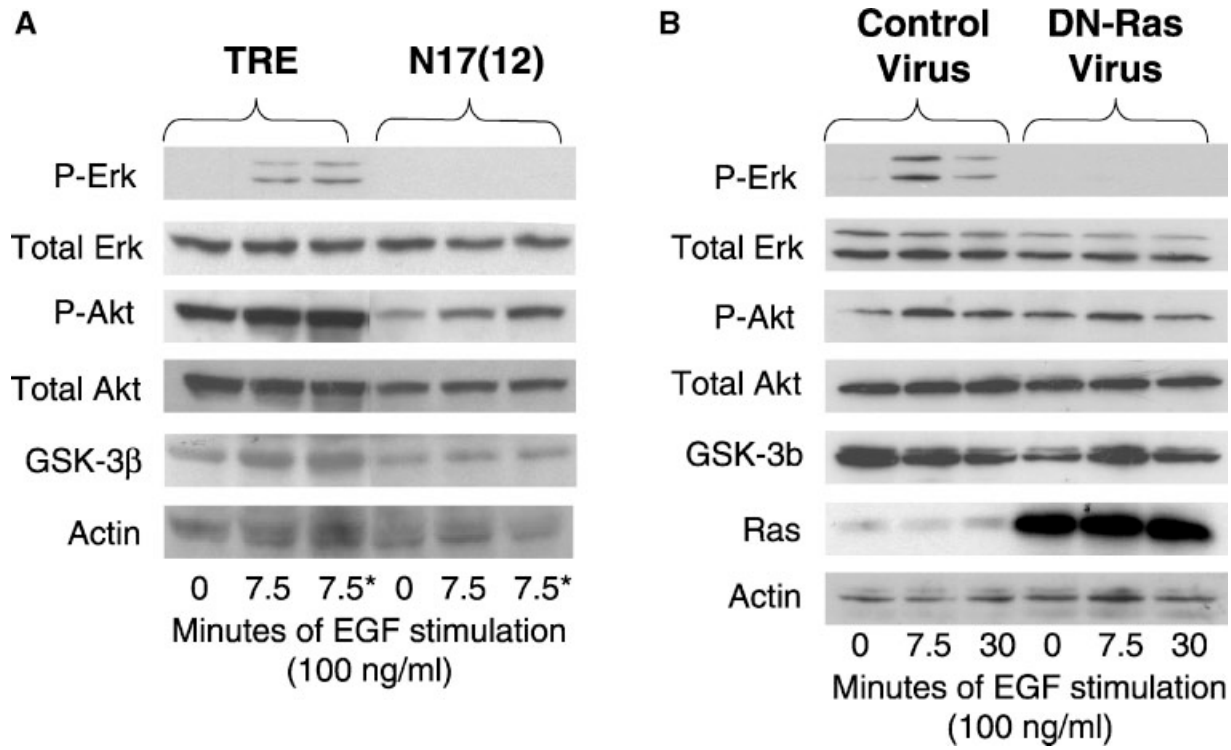


Fig. 7. The effect of DNRasN17 expression on signal transduction pathways in HC11 cells. **A**: The HC11 TRE vector control cells and DNRasN17 (clone 12) cell lines were grown to confluence in EGF-containing media lacking doxycycline. The cells were incubated in media without EGF or media without EGF and serum (*) prior to restimulation with EGF (100 ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting using antibodies specific for phosphorylated and nonphosphorylated forms of the indicated proteins. **B**: HC11 cells infected with control adenovirus vector or DNRasN17-encoding adenovirus at an MOI of 10 were incubated in serum-containing media for 24 h and incubated in EGF-free media for 20 h prior to stimulation with EGF (100 ng/ml) for the time indicated. Lysates of cells were harvested and analyzed by Western blotting as in part A.

with an antibody that recognizes the active phosphorylated forms of Erk1, 2. In contrast, in HC11 cells expressing DNRasN17 there was no activation of p44Erk, although the Erk protein levels in the cells were similar to those in the vector control cells. The analysis of other signaling proteins revealed little or no difference in Akt activation between the control HC11 cells and the DNRasN17 HC11 cells following treatment with EGF. This demonstrated that the PI-3-kinase pathway was not significantly blocked by DNRasN17 expression in HC11 cells. Moreover, activation of Jun kinase and p38 kinase by EGF was not deficient in the DNRasN17 HC11 cells (data not shown). These results suggest that the Mek–Erk pathway was most sensitive to inhibition by DNRas17 expression.

Cells infected with the control adenovirus vector or adenovirus encoding DNRasN17 were examined for the effect of EGF on signal transduction pathways in an analogous fashion. The results in Figure 7B demonstrated that DNRasN17 adenovirus also blocked the activation of Erk but not the phosphorylation of AKT on serine 473, used as a measure of PI-3-kinase activity. The results from the DNRasN17 expressing cells indicated that blocking the Ras pathway in this manner in HC11 cells primarily blocked signaling to the Raf–Mek–Erk pathway. Hence, these data support the conclusion that in HC11 cells activated RasV12 inhibits β-casein transcription via Mek–Erk signaling, and that the effect of DNRasN17 expression on β-casein is primarily a result of its inhibition of the Mek–Erk pathway.

Expression of dominant negative Ras prevents the prolactin-induced association of SHP2 with Stat5

Our results demonstrated that the expression of DNRasN17 resulted in enhanced DIP-induced activation of Stat5 as measured by tyrosine phosphorylation, DNA binding and activation of the β-casein promotor. To determine the mechanism by which this occurs, the functionality of several Stat5 regulatory pathways in DNRasN17 cells was examined. The increased activity of Stat5 likely resulted from the higher level of Stat5 tyrosine 694 phosphorylation; hence, regulation of Stat5 tyrosine phosphorylation was examined by looking for the association of tyrosine phosphatases with Jak2 and Stat5. Previous reports demonstrated that the phosphatase SHP2 interacted with Stat5; SHP2 associated with Stat5 following stimulation of the Stat5 pathway by prolactin as detected by co-immunoprecipitation of SHP2 with Stat5 (Chughtai et al., 2002). To determine if dominant negative Ras expression affected the prolactin-induced association of SHP2 with Stat5, HC11-TRE, and DNRasN17 cells were stimulated with prolactin. Cell lysates were prepared, Stat5 was immunoprecipitated, and the amount of SHP2 associated with the Stat5 was determined by Western blotting. The

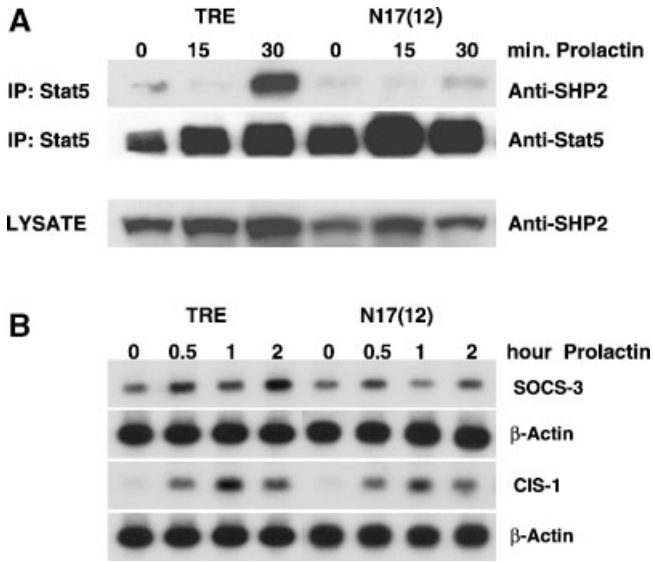


Fig. 8. Prolactin-induced binding of SHP2 to Stat5 and prolactin-induced expression of SOCS-3 and CIS-1. HC11-TRE and HC11-DNRasN17 (clone 12) cells were grown to confluency in EGF-containing media then incubated in EGF-free media for 24 h; the cells stimulated with prolactin (5 μg/ml) for the indicated time. **A**: Lysates were prepared and Stat5 was immunoprecipitated. The lysates and the immunoprecipitates were analyzed by Western blotting for Stat5 and SHP2. **B**: RNA was extracted from cells and analyzed by Northern blotting for expression of SOCS-3 and CIS-1. β-Actin was hybridized to the blots as a loading control.

results in Figure 8A demonstrated that the level of SHP2 protein expression was not reduced in HC11-DNRasN17 cells. However, in the vector control cell line, prolactin stimulation for 30 min resulted in significant association of SHP2 with Stat5, but very little SHP2 was associated with Stat5 in prolactin stimulated DNRasN17 cells (Fig. 8A). Accordingly, the degree of Stat5 phosphorylation at Y694 was significantly greater in the DNRasN17 cells than in the TRE cells.

In previous reports PTP-PEST was identified as a phosphatase that was activated by EGF and prolactin and associated with Jak2 (Horsch et al., 2001). These studies indicated that PTP-PEST was co-immunoprecipitated with Jak-2 from HC11 cells following prolactin stimulation. However, we did not observe this association in either the HC11-TRE or HC11-DNRasN17 cell lines (data not shown).

A previous report indicated that both EGF and prolactin stimulation induced the expression of the inhibitors of cytokine signaling, SOCS-3 and CIS-1, in HC11 cells (Tonko-Geymayer et al., 2002). To determine if changes in the regulation of SOCS-3 and CIS-1 expression was affected by dominant negative Ras expression, the level of SOCS-3 and CIS-1 expression was examined by Northern blotting at various times after the addition of prolactin to HC11-TRE and DNRasN17 cell lines. The results, shown in Figure 8B, indicated that the expression of CIS-1 and SOCS-3 was stimulated by prolactin and that the expression was similarly regulated in the HC11- DNRasN17 cells and the HC11-TRE control cell line.

These results indicated that the association of SHP2 tyrosine phosphatase activity with Stat5 was blocked by dominant negative Ras expression. The resulting block to Stat5 dephosphorylation constitutes a likely mechanism for the enhancement of Stat5 activation by DNRasN17 expression.

DISCUSSION

Members of the EGF family of peptide growth factors are found in the mammary gland and appear to play a role in growth and differentiation in that tissue (Jhappan et al., 1990). For example, EGF and amphiregulin are expressed in the ductal epithelial cells and TGFα is expressed in cap stem cells in the terminal end buds (Snedeker et al., 1992; Kenney et al., 1995). EGF and TGFα, bind to EGF receptor (ERB1) and can stimulate the proliferation of mammary epithelial cells and enhance lobular–aveolar development in the mammary gland of virgin mice (Vonderhaar, 1987). These growth factors can also prevent milk protein expression in HC11 cells and inhibit apoptosis of secretory alveolar epithelial cells in the involuting mammary gland (Smith et al., 1995). Hence, these factors play a dual role in mammary differentiation.

Growth factors of the EGF family have been detected in human breast tissue and elevated levels have been associated with breast tumors (Dotzlaw et al., 1990; Mizukami et al., 1991). The stimulation of mammary cells in culture by these growth factors activates signal transduction pathways that lead to cell survival and mitosis, and the activation of the EGF-R (ErbB1) correlates with aggressive behavior of breast tumors (Arteaga et al., 1988; Umekita et al., 1992). One of the signaling molecules activated by EGF family growth factors in breast tumors is the Ras GTPase (von Lintig et al., 2000). Previous studies have demonstrated that EGF and activated Ras inhibit differentiation in HC11 cells. Both stimulation of mammary epithelial cells with EGF and the expression of activated Ras initiate signaling through the Mek–Erk pathway. While EGF stimulation also leads to activation of the PI-3-kinase pathway, the influence of Ras on this pathway in HC11 cells has not been examined. Therefore, the present study analyzed the effects of EGF on the Ras, Erk, and PI-3-kinase pathways in HC11 cells and the contribution of those pathways to lactogenic hormone-induced differentiation.

The results confirmed the findings of several previous studies by demonstrating that EGF can block lactogenic hormone-induced differentiation in HC11 cells (Hynes et al., 1990). Chemical inhibitor studies indicated that the inhibition of β-casein promotor activity by EGF required both the Mek–Erk and PI-3-kinase pathways. While a previous study found that activation of the Erk pathway was not required for lactogenic differentiation (Wartmann et al., 1996), the contribution of Erk to the inhibition of lactogenic hormone-induced differentiation by EGF was less clear. Merlo et al. (1996) correlated the inhibition of lactogenic hormone-induced differentiation by growth factors with the ability of different growth factors to induce a high level of Erk activation. Also, expression of v-Raf, an activator of Mek–Erk signaling, inhibited lactogenic hormone-induced differentiation of HC11 cells (Happ et al., 1993). However, a

previous study by DeSantis et al. (1997) demonstrated that inhibition of Ras and PI-3-kinase blocked the inhibitory effects of EGF on β-casein synthesis. Our study extends this previous study and demonstrates that the inhibition of the Erk pathway strongly correlates with an increase in β-casein promotor activation. Moreover, in our study the stable expression of dominant negative Ki-RasN17 or the infection of HC11 cells with dominant negative Ha-RasN17 adenovirus effectively enhanced β-casein synthesis in response to lactogenic hormones, and these cells exhibited inhibition of the Mek–Erk pathway but not the PI-3-kinase signaling pathway. Hence, it appears that the Erk pathway is critical in the negative regulation of lactogenic hormone-induced differentiation by DNRas. This appears be a function of its effect on Stat5 tyrosine phosphorylation and activation. Our results are in agreement with those of Gao et al., which suggest that Erk activation alters prolactin-induced expression at a step prior to Stat5 DNA binding (Gao and Horseman, 1999). The HC11 cells expressing dominant negative Ras, which were defective in Erk activation, exhibited both an increase in Stat5 tyrosine phosphorylation and an increase in Stat5 DNA binding.

The SH2 protein tyrosine phosphatase, SHP2, has been identified in a complex with Stat5 and a role for this phosphatase in regulation of Stat5 activity has been proposed (Berchtold et al., 1998; Chughtai et al., 2002). Our results indicated that DNRas expression blocked the association of SHP2 with Stat5. The mechanism by which this occurs has not been resolved. In addition to involvement in Jak-Stat signaling, SHP2 is required for growth factor receptor activation of the Ras–Erk pathway. SHP2 plays an essential role in linking components of signal transduction pathways to growth factor receptor complexes via the scaffold protein Gab1, which targets SHP2 to the membrane (Cunnick et al., 2002). Two potential links for SHP2 to the Ras pathway have been reported. Dominant negative SHP2 expression decreased the level of activated Ras (Ras-GTP) in cells (Cai et al., 2002); this could result from a decrease in guanine nucleotide exchange factor (SOS) activity. Alternatively, it was recently reported that SHP2 regulated EGF-dependent RasGAP, but not SOS, membrane localization and increased the half-life of Ras-GTP (Agazie and Hayman, 2003). Our data demonstrated that dominant negative Ras expression, which interferes with Ras activation in part by binding and sequestering guanine nucleotide exchange factors (Lai et al., 1993), disrupts one aspect of SHP2 function. This suggests that there may exist a mechanism to regulate SHP2 by Ras via SOS or RasGAP.

While EGF stimulation of HC11 cells has been linked to the activation of PTP-PEST and dephosphorylation of Jak2 (Horsch et al., 2001), no association of PTP-Pest with Jak2 was detected following prolactin stimulation in the HC11-TRE or HC11-DNRasN17 cell lines. In addition, although expression of SOCS-3 and CIS-1 has been demonstrated in HC11 cells following exposure to prolactin (Tonko-Geymayer et al., 2002), DNRasN17 expression did not alter the transcriptional activation of SOCS-3 or CIS-1 following prolactin stimulation in our experiments. Collectively these results suggested that DNRasN17 expression enhanced Stat5 tyrosine phosphorylation primarily by blocking the association of the SHP2 phosphatase with Stat5.

The data presented here demonstrate that the addition of EGF to HC11 cells stimulates the PI-3-kinase pathway resulting in the phosphorylation of Akt and its downstream signaling pathway. The data also demonstrate that inhibition of the PI-3-kinase pathway increases β-casein promotor activity. The expression of dominant negative Ki-Ras did not prevent the activation of PI-3-kinase-Akt pathway, indicating that the activation of PI-3-kinase was primarily a consequence of the binding of the p85 subunit to the EGF receptor rather than the direct activation of p110 by activated Ras (Rodriguez-Vicania et al., 1994). These results suggest that the PI-3-kinase pathway influences a stage in Jak-Stat signaling that occurs prior to or at the level of DNA binding. A recent study has demonstrated that PI-3-kinase inhibition enhanced Stat5 activation by thrombopoitin in part by preventing nuclear export of Stat5 (Kirito et al., 2002).

There have been several studies in other tissues demonstrating that regulation of Ras-dependent signal transduction contributes to differentiation. For example, there is evidence from both in vivo systems and tissue culture systems that the Ras–Raf–Mek–Erk pathway is required for neuronal differentiation (Halegoua et al., 1991; Thomas et al., 1992; Wood et al., 1992; Cowly et al., 1994; Marshall, 1995). Also, the activation of the Mek–Erk pathway may contribute to the differentiation status of some breast cancer cell lines. For example, differentiation-linked Erk activation in breast cancer cells occurs following ligand-induced activation of RTKs, including stimulation with heregulin (NDF, Neu differentiation factor) and subsequent activation of HER-3 (Lessor et al., 1998), or following transfection and overexpression of c-erbB-2 (Giani et al., 1998). In both systems activation of the Ras–Erk pathway resulted in increased expression of $p21^{CIP}$ and enhanced differentiation. ErbB4 signaling has also been linked to prolactin-induced Stat5 activation (Jones et al., 1999). Hence, because of dual nature of Mek–Erk signaling in differentiation, it is important to understand the role of the Ras pathway in lactogenic hormone-induced differentiation. The results of this study clearly focus on signaling through the Mek–Erk pathway as a Ras-regulated disruptor of lactogenic hormone-induced differentiation. Moreover, by identifying the Mek–Erk pathway along with altered regulation of SHP2 as pathways that are inhibited by DNRasN17, these studies suggest an additional mechanism by which EGF disrupts differentiation in this cell line.

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LITERATURE CITED

Agazie Y, Hayman M. 2003. Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling. Mol Cell Biol 23: 7875–7886.

Akasaka K, Tamada M, Wang F, Kariya K, Shima F, Kikuchi A, Yamamoto M, Shirouzu M, Yokoyama S, Katoaka T. 1996. Differential structural requirements for interaction of Ras protein with its distinct downstream effectors. J Biol Chem 271:5353–5359.

Ali S. 1998. Prolactin receptor regulates Stat5 tyrosine phosphporylation and nuclear translocation by two separate pathways. J Biol Chem 273(13):7709–7716.

Arteaga C, Hanauske A, Clark G, Osborne C, et al. 1988. Immunoreactive alpha transforming growth factor in effusions from cancer patients as a marker of tumor burden and patient's prognosis. Cancer Res 48:5023–5028.

Bacus S, Huberman E, Chin D, Kiguchi K, Simpson S, Lippman M, Lupu R. 1992. A ligand for the ErbB2 oncogene product (gp30) induces differentiation of human breast cancer cells. Cell Growth Differ 3:401–411.

Ball R, Friis R, Schonenberger C, Doppler W, Groner B. 1988. Prolactin regulation of a β-casein gene expression and a cytosolic 120 kDa protein in a cloned mouse mammary epithelial cell line. EMBO J 7:2089–2095.

Berchtold S, Volarevic S, Morrigl R, Mercep M, Groner B. 1998. Dominant-negative variants of SHP-2 tyrosine phosphatase inhibit prolactin activation of Jak2 and induction of Stat5-dependent transcription. Mol Endocrinol 12:556–567.

Blatchford D, Hendry K, Turner M, Burgoyne R, Wilde C. 1995. Vectorial secretion by constitutive and regulated secretory pathways in mammary epithelial cells. Epithelial Cell Biol 4:5–8.

Cai T, Nishida K, Hirano T, Khavari PA. 2002. Gab1 and SHP-2 promote Ras/MAPK regulation of epidermal growth and differentiation. J Cell Biol 159(1):103–112.

Chughtai N, Schimchowitsh S, Lebrun J, Ali S. 2002. Prolactin induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta-casein gene promotor. J Biol Chem 277(34): 31107–31114.

Cowly S, Paterson H, Kemp P, Marshall C. 1994. Activation of Map kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH3T3 cells. Cell 77:841–852.

Cunnick JM, Meng S, Ren Y, Desponts C, Wang HG, Djeu JY, Wu J. 2002. Regulation of the mitogen-activated protein kinase signaling pathway by SHP2. J Biol Chem 277(11):9498–9504.

Danielson K, Oborn C, Durbam E, Butel J, Medina D. 1984. Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc Natl Acad Sci 81:3756–3760.

DeSantis M, Kannan S, Smith G, Seno M, Bianca C, Kim N, Martinez-Lacaci I, Wallace-Jones B, Salomon D. 1997. Cripto-1 inhibits β-casein expression in mammary epithelial cells through a p21Ras- and phosphatidylinositol 3′-kinase-dependent pathway. Cell Growth Differ 8:1257–1266.

Dotzlaw H, Miller T, Karvelas J, Murphy L. 1990. Epidermal growth factor gene expression in human breast biopsies samples: Relationship to estrogen and progesterone receptor gene expression. Cancer Res 50:4204–4208.

Gao J, Horseman N. 1999. Prolactin-independent modulation of the beta-casein response element by Erk2 Map kinase. Cell Signaling 11:205–210.

Giani C, Casalini P, Pupa S, DeVecchi R, Ardini E, Colnaghi M, Giordano A, Menard S. 1998. Increased expression of c-erbB-2 in hormone-dependent breast cancer cells inhibits cell growth and induces differentiation. Oncogene 17:425–432.

Gouilleux F, Wakeo H, Mundt M, Groner B. 1994. Prolactin induces phosphorylation of Tyr 694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J 13:4361–4369.

Halegoua S, Armstrong R, Kremer N. 1991. Dissecting the mode of action of a neuronal growth factor. Curr Top Microbiol Immunol 165:120–170.

Han Y, Watling D, Rogers N, Stark G. 1997. Jak2 and Stat5, but not Jak1 and Stat1, are required for prolactin-induced beta-lactoglobulin transcription. Mol Endocrinol 11:1180–1188.

Happ B, Hynes N, Groner B. 1993. Ha-Ras and v-Raf oncogenes, but not int-2 and c-myc, interfere with lactogenic hormone dependent activation of the mammary gland specific transcription factor. Cell Growth Differ 4(1):9–15.

Horsch K, Schaller M, Hynes N. 2001. The protein tyrosine phosphatase-PEST is implicated in the negative regulation of epidermal growth factor on PRL signaling in mammary epithelial cells. Mol Endocrinol 15(12):2182–2196.

Humphreys R, Rosen J. 1997. Stably transfected HC11 cells provide and in vitro and in vivo model system for studying Wnt gene expression. Cell Growth Differ 8:839–849.

Hynes N, Taverna D, Harwerth I, Ciardiello F, Salomon D, Yamamoto T, Groner B. 1990. Epidermal growth factor, but not c-erb-2, activation prevents lactogenic hormone induction of β-casein gene in mouse mammary epithelial cell line. Mol Cell Biol 10:4027–4034.

Janes P, Daly R, DeFazio A, Sutherland R. 1994. Activation of the Ras signaling pathway in human breast cancer cells overexpressing erbB-2. Oncogene 9:3601–3608.

Jehn B, Costello E, Marti A, Keon N, Deane R, Li F, Friis R, Burri P, Martin F, Jaggi R. 1992. Overexpression of Mos, Ras, Src, and Fos inhibits mouse mammary epithelial cell differentiation. Mol Cell Biol 12(9):3890–3892.

Jhappan C, Stahle C, Harkins R, Fausto N, Smith G, Merlino G. 1990. TGFalpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and the pancreas. Cell 61:1137–1146.

Jones F, Welte T, Fu X-Y, Stern D. 1999. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol 147:77–87.

Kenney N, Huang R-P, Johnson G, Wu J-X, Okamura D, Matheny W, Kordon E, Gullick W, Plowman G, Smith G, Salomon D, Adamson E. 1995. Detection and location of amphiregulin and cripto-1expression in the developing postnatal mouse mammary gland. Mol Reprod Dev 41:277–286.

Kikuchi A, Demo S, Ye Z, Chen Y, Williams L. 1994. Ral GDS family members interact with the effector loop of Ras p21. Mol Cell Biol 14:7483–7491.

Kirito K, Watanabe T, Sawada K, Endo H, Ozawa K, Komatsu N. 2002. Thrombopoitin regulates Bcl-xL gene expression through Stat5 and phophatidylinositol 3-kinase. J Biol Chem 277:8329–8337.

Lai CC, Boguski M, Broek D, Powers S. 1993. Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins. Mol Cell Biol 13:1345–1352.

Lessor T, Yoo J-Y, Davis M, Hamburger A. 1998. Regulation of heregulinb1-induced differentiation in a human breast carcinoma cell line by the extracellular-regulated kinase pathway. J Cell Biochem 70:587–595.

Marshall C. 1995. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185.

Marte B, Jeschke M, Grause-Porta D, Taverna D, Hofer P, Groner B, Yarden Y, Hynes N. 1995. Neu differentiation factor/heregulin modulates growth and differentiation of HC11 mammary epithelial cells. Mol Endocrinol 9:14–23.

Masuelli L, Ettenberg S, Vasaturo F, Vestergaard-Sykes K, Cutler M. 1999. The Ras suppressor, Rsu-1, enhances NGF-induced differentiation of PC12 cells by induction of p21CIP. Cell Growth Differ 10:555–564.

Merlo G, Grause-Porta D, Cella N, Marte B, Taverna D, Hynes N. 1996. Growth, differentiation, and survival of HC11 mammary epithelial cells: Diverse effects of receptor tyrosine kinase-activating peptide growth factors. Eur J Cell Biol 70:97–105.

Mizukami Y, Nonomura A, Noguchi M, Taniya T, et al. 1991. Immunohistochemical study of oncogene product Ras p21, c-myc, and growth factor EGF in breast carcinomas. Anticancer Res 11:1485–1494.

Moodie S, Willumsen B, Weber M, Wolfman A. 1993. Complexes of Ras-GTP with Raf-1 and mitogen activated protein kinase. Science 260:1658–1661.

Nicke B, Tseng M-J, Fenrich M, Logsdon C. 1999. Adenovirus-mediated gene transfer of Ras N17 inhibits specific CCK actions on pancreatic acinar cells. Am J Physiol 276:G499–G506.

Peterson H, Haldosen L. 1998. EGF modulates expression of Stat5 in mammary epithelial cells. Exp Cell Res 243:347–358.

Rodriguez-Vicania P, Warne P, Dhand R, Vanhaesebroek B, Gout I, Fry M, Waterfield M, Downward J. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 370:527–532.

Salomon D, Bianco C, DeSantis M. 1999. Cripto: A novel epidermal growth factor (EGF)-related peptide in mammary gland development and neoplasia. BioEssays 21:61–70.

Shibuya E, Polverino A, Chang E, Wigler M, Ruderman J. 1992. Oncogenic Ras triggers the activation of 42-kDa mitogen-activated protein kinase in extracts of quiscent *Xenopus* oocytes. Proc Natl Acad Sci 89:9831–9835.

Smith G, Sharp R, Kordon E, Jhappan C, Merlino G. 1995. Transforming growth factor alpha promotes mammary tumorigenesis through selective survival and growth of secretory epithelial cells. Am J Pathol 147:1081–1096.

Snedeker S, Brown C, DiAugustine R. 1992. Expression and functional properties of transforming growth factor alpha and epidermal growth factor during mouse mammary ductal morphogenisis. Proc Natl Acad Sci 88:276–280.

Taverna D, Groner B, Hynes N. 1991. Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-ErbB receptor activation all promote growth but have distinctive effects upon mouse mammary epithelial cell differentiation. Cell Growth Differ 2:145–154.

Thomas S, DeMarco M, D'Arcangelo G, Halegoua S, Brugge J. 1992. Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. Cell 68:1031–1040.

Tonko-Geymayer S, Goupille O, Tonko M, Soratroi C, Yoshimura A, Streuli C, Ziemiecki A, Kofler R, Doppler W. 2002. Regulation and function of the cytokine-inducible SH-2 domain proteins, CIS and SOCS3, in mammary epithelial cells. Mol Endocrinol 16(7):1680–1695.

Umekita Y, Enokizono N, Sagara Y, Kuriwaki K, et al. 1992. Immunohistochemical studies on oncogene products (EGF-R, c-erbB2) and growth factors (EGF, TGFalpha) in human breast cancer: Their relationship to oestrogen receptor status, histological grade, mitotic index, and nodal status. Virchows Arch A Pathol Anat Histopathol 420:345–351.

Van Aelst L, Barr M, Marcus S, Polverino A, Wigler M. 1993. Complex formation between Ras and Raf and other protein kinases. Proc Natl Acad Sci 90:6213–6217.

von Lintig F, Dreilinger A, Varki N, Wallace A, Casteel D, Boss G. 2000. Ras activation in human breast cancer. Breast Cancer Res Treat 62(1):51–62.

Vonderhaar B. 1987. Local effect of EGF, TGFalpha, and EGF-like growth factors on lobuloalveolar development in the mouse mammary gland in vivo. J Cell Physiol 132:581–584.

Warne P, Viciana P, Downward J. 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 364:352–355.

Wartmann M, Cella N, Hofer P, Groner B, Liu X, Henninghousen L, Hynes N. 1996. Lactogenic hormone activation of Stat5 and transcription of the β-casein gene in mammary epithelial cells is independent of p42Erk mitogen-activated protein kinase activity. J Biol Chem 271:31863–31868.

Wood K, Sarnecki C, Roberts T, Blenis J. 1992. Ras mediates nerve growth factor receptor modulation of three signal-transducting protein kinases: Mapkinase, Raf-1, and RSK. Cell 68:1041–1050.

Wyszomierski S, Yeh J, Rosen J. 1999. Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation. Mol Endocrinol 13(2):330–343.

EGF stimulation of the PI-3-K pathway activates p70S6 Kinase during inhibition of HC11 lactogenic differentiation

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Abbreviations: EGF; epidermal growth factor, PI-3-K; phosphatidylinositol-3-kinase, DIP; dexamethasone, insulin and prolactin, PRL; prolactin, MECs; mammary epithelial cells, DN; dominant negative, CA; conditionally active, PIP; prolactin inducible protein, OPN; osteopontin

Abstract

Epidermal growth factor (EGF) stimulation of mammary epithelial cells (MECs) inhibits differentiation and apoptosis. EGF can activate the phosphatidylinositol–3-kinase (PI-3-K) pathway in MECs, and breast tumors with constitutive PI-3-K activation exhibit resistance to chemotherapy. Therefore, the dissection of this pathway in normal MECs is critical to the understanding of signaling events that impact breast cancer outcome. HC11 mouse MECs differentiate in response to lactogenic hormone resulting in expression of milk proteins including β-casein. Previous studies have shown that EGF blocks differentiation through activation of the Ras/Mek/Erk pathway but also implicated PI-3-K signaling. Therefore, specific chemical inhibitors of signal transduction pathways, adenovirus encoding dominant negative-Akt (DN-Akt) and a plasmid expressing conditionally active-Akt (CA-AKT) were used to analyze the role of the PI-3-K pathway in an EGF-induced block of HC11 differentiation. The expression CA-Akt inhibited lactogenic differentiation of HC11 cells. In contrast, treatment with LY294002 or infection with DN-Akt enhanced β-casein transcription and rescued β-casein promotor driven luciferase activity in the presence of EGF, demonstrating that the EGF block of HC11 lactogenic differentiation is, in part, PI-3-K/Akt dependent. Inhibition of PI-3-K modestly enhanced Stat5 phosphorylation. Investigation of pathways downstream of PI-3-K using inhibitors of mTOR (Rapamycin) and p38 stress kinase (SB203850) revealed that both treatments blocked the effects of EGF on β-casein promotor driven luciferase activity as effectively as PI-3-K inhibitors. Moreover, the inhibition of either PI-3-K or mTOR abolished the activation of p70S6 Kinase (p70S6K) by EGF in HC11 cells, and expression of CA-Akt in HC11 cells caused a constitutive activation of p70S6K. Thus,

PI-3-K signaling via Akt and p70S6K contributes to the EGF block of lactogenic differentiation. Additional investigation determined that activation of p70S6K resulted in the phosphorylation of RPS6, eIF4E and 4E-BP1 via PI-3-K dependent mechanisms. Following DIP-stimulation of HC11 cells the activation of p70S6K completely diminished by approximately 12 hours, whereas stimulation with DIP in the presence of EGF p70S6K activation persisted for 24 hours. In contrast, the cells treated with LY294002 showed no p70S6K activation at any time point after induction. This data suggests that the EGF-induced activation of PI-3-K ultimately influences translational control of proteins involved in the regulation of lactogenic differentiation.

Introduction

Up to 30% of all breast tumors exhibit elevated expression of one or more members of the epidermal growth factor receptor family (ErbB), and these molecules are common targets for breast cancer therapy (LeVea, 2004; Reise, 1998; Danielsen, 2002). Excess activation of signaling pathways downstream of the epidermal growth factor receptor has been directly linked to breast cancer development and chemotherapeutic resistance (Navolanic, 2003). While epidermal growth factor is required for normal mammary epithelial cell proliferation, it has been shown to inhibit lactogenic differentiation of mammary epithelial cells both *in vitro* and *in vivo* (Hynes, 1990; Brandt, R., 2001; Cerrito, 2004).

HC11 mouse mammary epithelial cells have been widely used as an *in vitro* model of mammary gland epithelial cell differentiation (Hynes, 1990; Cerrito, 2004; Petersen, 1998; Bailey, 2004). The HC11 cell line, clonally derived from the COMMA-1D line obtained from mid-pregnant BALB/c mice, preserves important features of mammary epithelial cell lactogenic differentiation (Ball, 1988; Hynes, 1990). The cells are non-tumorigenic, display a normal phenotype, and the injection of HC11 cells into the cleared fat pad of BALB/c mice exhibited normal ductal and alveolar-like structures (Humphreys, 1997). HC11 mammary epithelial cell lactogenic differentiation can be initiated in culture following the growth to confluence in the presence of epidermal growth factor, followed by the removal of epidermal growth factor from the culture and the addition of lactogenic hormone mix DIP (dexamethasone, insulin, and prolactin). Numerous studies have shown that HC11 cells differentiate in response to lactogenic hormone and express specific milk proteins including β-casein and WAP (Hynes, 1990;

Merlo, 1996; Bailey, 2004). HC11 cells express receptor tyrosine kinases of various subclasses (Merlo, 1996; Marte, 1994; Cerrito, 2004; Bailey, 2004), and the addition of mitogens (epidermal growth factor, fibroblast growth factor) or the presence of oncogenes (activated Ras) inhibit lactogenic differentiation (Hynes, 1990; Merlo, 1996; Tonko-Geymayer, 2002; Petersen, 1998; Cerrito, 2004). Several signaling pathways have been shown to facilitate the epidermal growth factor-induced block, however the mechanisms have not been fully elucidated. The two key pathways implicated are Ras/Mek/Erk and phosphatidylinositol-3-kinase (PI-3-K) pathways (Petersen, 1998; Ebert, 1999; Merlo, 1996). Our previous study demonstrated (Cerrito, 2004) that DN-Ras expression blocked epidermal growth factor induced inhibition of HC11 cell lactogenic differentiation via inhibition of Raf/Mek/Erk signaling. However, the activation of PI-3-K by EGF was largely independent of Ras in these cells (Cerrito, 2004).

The PI-3-Kinases are an ubiquitously expressed enzyme family that has lipid kinase activity and plays a key role in cellular proliferation, growth and survival (Wendel, 2004; Fingar, 2002). PI-3-K was purified and cloned as a heterodimeric complex consisting of an 110kDa catalytic subunit and an 85kDa regulatory/adaptor subunit (Carpenter, 1996). PI-3-K is activated following either binding of the p110 subunit to activated Ras or by the SH2 domains of the p85 adaptor protein binding to the phosphotyrosine residues of the epidermal growth factor receptor (Cantley, 2002; Navolanic, 2003). EGF induces receptor dimerization and subsequent autophosphorylation of Tyr residues in the cytoplasmic tail of the receptor to create a docking site for SH2-domain interaction. Kinase activation is the consequence of translocation of PI-3-K from the cytosol to the

membrane where its substrate is located (Cantley, 2002; Navolanic, 2003). PI-3-K phosphorylates the 3'-OH position of the inositol ring of phosphatidylinositol-4, 5-bisphosphate (Fruman, 1998). This phosphorylation directs the membrane localization of PDK1 through its pleckstrin homology domain resulting in the autophosphorylation of PDK1 (Vivanco, 2002). Activation of PDK1 in turn phosphorylates Akt. Akt can be phosphorylated at two different sites. The first is at the Threonine (Thr) 308 and the second is at Serine (Ser) 473 (Sarbassov, 2005). PDK1 phosphorylates Akt at Thr 308 (Vivanco, 2002). Maximal activation of Akt requires Ser 473 to be phosphorylated by a kinase that has yet to be completely characterized referred to as PDK2 (Vivanco, 2002). There are at least 13 substrates identified for Akt including mTOR, GSK3β, p70S6 Kinase (p70S6K), cyclin D1, and FKHR1; however the mechanism by which PI-3-K/Akt contributes to mammary carcinogenesis has not been fully elucidated (Shen, 2003). Therefore, the dissection of the PI-3-K pathway is critical to our understanding of normal epithelial cell signaling as well as signaling tumor cells.

In the present study, we have addressed the mechanism by which PI-3-K blocks lactogenic differentiation in HC11 mammary epithelial cells. We determined that expression of conditionally active-Akt blocks lactogenic differentiation. By using chemical inhibitors of PI-3-K as well as a dominant negative-Akt we confirmed that blocking PI-3-K rescues the epidermal growth factor block of lactogenic differentiation in HC11 mammary epithelial cells. Epidermal growth factor stimulation activates Akt, p70S6K, Ribosomal protein S6 (RPS6), eIF4E and 4E-BP1 via PI-3-K/Akt dependent mechanisms in HC11 cells. Therefore, our data suggest that activation of PI-3-K

regulates changes in translational control of proteins that inhibit lactogenic differentiation in HC11 mammary epithelial cells.

Materials and Methods

Cell Culture:

Mouse mammary epithelial cell (MEC) lines, HC11 and HC11-luci, were a generous gift from Dr. Nancy Hynes (Taverna, 1991; Hynes, 1990). The HC11-luci cell line contains a luciferase gene under the control of a β-casein promotor (Wartmann, 1996). They were maintained in RPMI 1640 medium (Biosource) augmented with 10% fetal bovine serum (FBS) (Biosource), 5μg/ml Insulin (Biosource), 10ng/ml epidermal growth factor (EGF) (Calbiochem), 10mM HEPES (Biosource), Pen-Strep (Biosource), and 2mM Glutamine (Biosource) referred to as regular growth media (Cerrito, 2004). HEK-293 cells (ATCC) used for virus propagation were maintained in DMEM medium (Biosource) augmented with 10% FBS, Pen-Strep, and 2mM Glutamine.

Lactogenic hormone induced differentiation:

The technique for lactogenic differentiation of HC11 cells was decribed previously (Cerrito, 2004). Briefly, HC11 and HC11-luci cells were grown to confluence and maintained 1-3 days in RPMI 1640 augmented with 10% FBS, 5μg/ml Insulin, 10ng/ml EGF, 10mM HEPES, Pen-Strep, and 2mM Glutamine. EGF containing media was removed and cells were rinsed with media containing 10% FBS and lacking EGF. The cells were incubated in RPMI differentiation media containing either 1% FBS or 10% FBS, dexamethasone (10^{-6}M), 5μg/ml Insulin, and 5μg/ml ovine prolactin (PRL)(Sigma) referred to as DIP. The cells were harvested and processed at stated times and using stated procedures. HC11 differentiation was characterized by mammosphere formation and β-casein transcription. Mammospheres were enumerated by microscope observation

and photographed at 96 hours post-induction, and the number of mammospheres per field was reported (Blatchford, 1999; Xie, 2002). β-casein transcription was assessed via northern Blotting or real time PCR. HC11-luci lactogenic differentiation was characterized via β-casein promotor driven luciferase activity.

Transfection of cell line:

The HC11 and HC11-luci cells were transiently transfected with either a conditionally active-Akt-1 (myrΔ4-129-ER or referred to CA-Akt in the paper) or a control construct (pCDNA3.1), which were generously provided by Dr. Richard Roth (JBC, 1998). The conditionally active-Akt-1 was created by attaching a *src* myristoylation signal to the amino terminus of a variant Akt that lacked its PH domain and carried an HA epitope tag at its carboxyl terminus. This was then fused in frame to the hormone-binding domain of a mutant form of the murine estrogen receptor therefore making it responsive to the synthetic steroid hydroxy-tamoxifen (Kohn, 1998). The cells were transfected at 80% confluence in 35mm wells with 3μg of plasmid DNA and Gene Juice (Novagen) as recommended by manufacturer.

Adenovirus propagation, titration and infection:

25 x T-175 flasks of 293 cells were grown to 90% confluence and infected with either a replication defective Lac Z control adenovirus or DN-Akt1 (DN-Akt) adenovirus kindly provided by Dr. Kenneth Walsh (JBC, 1999). The DN-Akt1 vector is a triple-A mutant because it contains alanine substitutions at the active site (residue179) as well as both regulatory phosphorylation sites (Thr308, Ser473). It also contains a HA-Tag at its N-terminus (Fujio, 1999). Cells were harvested 48 hours later, pelleted and re-suspended

in 8 mls of PBS. The samples went through four freeze-thaw cycles and were then purified via a cesium chloride gradient and dialyzed against a buffer containing 10mM Tris, 2mM $MgCl_2$, 100mM NaCl and 5% Glycerol. For titration, 293 cells were set-up at 1.5 x 10^5 cells per well and infected with serial dilutions of virus ranging from 10^{-2} to 10^{-8} in 1 ml of DMEM media. Cells were infected at 85% confluence for 5-6 hours and cytopathic effect (CPE) was assessed at 24 and 48 hours. HC11 and HC11-luci cells were infected with either the Lac Z control adenovirus or DN-Akt1 adenovirus at MOI of 10. After 5 hours virus was removed, regular growth media was added and cells were incubated 16-24 hours prior to treatment.

Luciferase assays:

The luciferase technique was previously described (Cerrito, 2004). Briefly, HC11 cells containing a luciferase gene under the control of a β-casein promotor (HC11-luci) were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media in the presence or absence of EGF (10ng/ml). Inhibitors were added alone or in combination at the time of induction at previously determined optimal concentrations (LY294002 10μM, SB203580 10μM, Rapamycin 50nM, PD98059 20μM) (data not shown). Luciferase activity was determined 48 hours post-induction using a commercial luciferase kit (Luciferase Assay Systems, Promega) and a Thermolab System luminometer (Acscent FL). Luciferase activity was normalized to protein as determined by BCA assay (Pierce, Rockford, IL). Results were presented as relative units calculated from the mean of three determinations.

Immunoprecipitations and Western blots:

HC11 cell lysates were lysed in either RIPA buffer (1% NP40, 0.5% DOC, 0.1% SDS, 150 mM NaCl, 5mM $MgCl_2$ and 25mM Hepes) or a high salt buffer (Wyszomierski, 1999). Each lysis buffer contained AEBSF (20μg/ml), aprotinin (5μg/ml), leupeptin (5μg/ml), β-glycerol phosphate (100μM), and $NaVAO_4$ (1mM). The following immunoprecipitation technique was described (Wyszomierski, 1999). Briefly immunoprecipitates for Stat5 were lysed in 200μl of high salt buffer, protein concentration was confirmed via BCA assay and 400μg was incubated in 0.5μg of primary antibody for two hours on ice. The protein was collected by adding 40μl of a 50% solution of Protein A agarose beads (Invitrogen Life Technologies) to each sample and incubating for one hour on a rocker at 4°C. The lysates were then pelleted at 5,000RPM for 1 minute, supernatant was removed and the Protein A beads were rinsed three times in RIPA buffer. For western blots equivalent amounts of protein were separated by SDS-PAGE and transferred to PVDF filters. The filters were blocked in 0.6% Blotto for one hour and then incubated with the appropriate primary antibody for one hour at room temperature or overnight at 4°C on a rocker. Blots were then incubated with appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. Blots were then washed three times for 10 minutes in TBST. Chemiluminescence was detected with either ECL (Amersham) or Supersignal (Peirce). Blots were then exposed to Classic Blue Sensitive x-ray film (Midwest Scientific). All blots were quantitated via scanning densitometry (Fuji, Image gauge software). Antibodies include anti-Stat5, sc-835 (Santa Cruz Biotech), and anti-phospho Stat5 Tyr694 (Cell Signaling Technology), anti-phospho (Ser 473 and Thr 308) and anti-AKT (Cell Signaling Technology), anti-

phospho-GSK3β (Cell Signaling Technology), anti-phospho Stat5 (S726/731) (Upstate), anti-phospho ERK (Cell signaling Technology), anti-ERK 1(Santa Cruz Biotechnology), anti-phospho p38 (Cell Signaling Technology), anti-p38 (Santa Cruz Biotechnology), anti-phospho JNK (Cell Signaling Technology), anti-JNK (Santa Cruz Biotechnology), anti-phospho p70S6Kinase (Thr389) (Cell Signaling Technology), anti-p70S6Kinase (Cell Signaling Technology), anti-phospho eIF4E (Ser289) (Cell Signaling Technology), anti-phospho 4E-BP1 (Ser65) (Cell Signaling Technology), anti-phospho Ribosomal protein S6 (Ser235/236) (Cell Signaling Technology), anti-phospho MNK1 (Thr197/202), anti-Pan Ras (Calbiochem), anti-β-Actin (clone AC-15) (Sigma) and anti-HA (clone 12CA5) (Roche). Antibodies were used at manufacturer's dilution recommendation.

Northern blot:

Total cell RNA was extracted using TriPure reagent (Roche). Northern Blots were prepared using 7.5ug of RNA, separated on a 1% agarose-formaldehyde gel, and transferred to a nylon filter. Blots were hybridized as described previously (Cerrito et al, 2004). The probes used were: mouse β-casein, mouse PIP (supplemental data), mouse OPN (supplemental data) and mouse β-Actin. Mouse β-casein probe is a 601 bp fragment (nucleotide 3-603) from the mouse β-casein cDNA, accession number XO4490.1. Mouse PIP is a 390 bp fragment (nucleotide 77-466) from mouse PIP cDNA, accession number NM_008843. Mouse OPN is a 498 bp fragment (nucleotide 829-1326) from mouse OPN cDNA, accession number NM_009263.

Results

EGF blocks HC11 lactogenic differentiation via Mek/Erk and PI-3-K dependent pathways

Our recent publication (Cerrito, 2004) as well as other reports suggest that PI-3-K plays a key role in mammary epithelial cell proliferation, survival, and important for this study, the block of lactogenic differentiation. The present study addresses the mechanism by which PI-3-K blocks HC11 mammary epithelial cell lactogenic differentiation. Several markers of HC11 mammary epithelial cell differentiation resulting from exposure to dexamethasone, insulin and prolactin (DIP) were used including β-casein synthesis (Hynes, 1990), Stat5 phosphorylation (Petersen, 1998) and mammosphere formation (Blatchford, 1999). We employed the use of two related cell lines to answer the above question. HC11 mammary epithelial cells, which were clonally derived from mid-pregnant BALB/c mice (Ball, 1998; Hynes, 1990) and HC11-luci cells which contain a luciferase gene under the control of a β-casein promotor (Wartmann, 1996).

EGF stimulation of HC11 cells activates PI-3-K signaling as well as other pathways and the addition of EGF to the lactogenic hormone cocktail prevents HC11 lactogenic differentiation (Hynes, 1990; Tonko-Geymayer, 2002; Horsch, 2001; Merlo, 1996). To more completely characterize the pathways activated by EGF and their impact on differentiation HC11-luci cells were induced to differentiate in DIP-lactogenic induction media in the presence or absence of EGF and specific inhibitors. Inhibitors were added singly at the time of induction at previously determined optimal concentrations. Luciferase activity was determined 48 hours post-induction. Luciferase activity was

normalized to protein concentration. As predicted, DIP stimulation induced β-casein driven luciferase expression, and EGF blocked the effect of DIP in HC11-luci cells (Figure 1A). Chemical inhibitors specific for the Mek/Erk pathway (PD98059) and PI-3-K pathway (Wortmannin and LY294002) rescued the cells from the EGF block of differentiation (Figure 1A). This result illustrates that EGF blocks HC11 lactogenic differentiation via Mek/Erk and PI-3-K dependent mechanisms. We examined β-casein RNA transcription in the presence of DIP, DIP + EGF and DIP + EGF + LY294002 (Figure 1B). HC11 cells were induced to differentiate in DIP-induction media with or without EGF and LY294002. Total cell RNA was harvested at 48 hours after induction and β-casein transcription was monitored by northern blotting. EGF blocked lactogenic hormone induced β-casein transcription and the addition of the PI-3-K inhibitor rescued β-casein transcription (Figure 1B). This finding confirmed our previous result (Cerrito, 2004).

Previous studies suggested that mammosphere formation is another important marker of HC11 lactogenic differentiation (Blatchford, 1999; Xie, 2002). We examined the effect of EGF on the formation of mammospheres. HC11 cells were induced to differentiate in DIP-induction media. EGF and LY294002 alone or in combination were added to designated samples at the time of induction. The cells were observed and photographed at 96 hours post-induction. The number of mammospheres was determined by counting the number of mammospheres per low power field and determining the mean of five fields. EGF completely blocked the formation of mammospheres and LY294002 not only rescued the EGF block of mammosphere formation, it enhanced the formation

over the DIP control (Figure 1C). This suggested that PI-3-K activation was important in blocking phenotypic lactogenic differentiation.

Constitutive activation of Akt-1 blocks lactogenic differentiation and the expression of dominant negative-Akt enhances differentiation

The activation of Akt is a major outcome of PI-3-K stimulation. Hence, the role of Akt in regulating HC11 lactogenic differentiation was examined. Transient transfection of a plasmid encoding a HA-tagged conditionally active-Akt-1 (HA-CA-Akt) gene was used to examine the role of the Akt pathway in blocking lactogenic differentiation via inhibition of β-casein promotor luciferase activity. HC11-luci cells were grown to 80% confluence and transfected with either a plasmid encoding a HA-tagged conditionally active-Akt-1 or a control vector. Western blot of transfected cells revealed that the HA-tagged conditionally active-Akt-1 was expressed at levels equal to the endogenous protein in the transfected HC11-luci cells. The cells were induced to differentiate with the addition of tamoxifen, which activates the kinase activity of HA-tagged conditionally-Akt-1, and harvested 48 hours after induction. The HA-tagged conditionally active-Akt-1 significantly decreased luciferase activity compared to the control (Figure 2).

Infection with a replication defective adenovirus encoding a dominant negative-Akt-1 (DN-Akt) was also used to further assess the role of the Akt pathway in blocking lactogenic differentiation. HC11 and HC11-luci cells were grown to 90% confluence and infected with a dominant negative-Akt-1 or a control adenovirus. At 24 hours post infection the cells were induced to differentiate and then harvested at either 24 or 48 hours post-induction. DN-Akt was assessed by western blotting and β-casein promotor

luciferase activity (Figure 3A) and β-casein RNA expression (Figure 3B) was determined. Infection with the dominant negative-Akt1 adenovirus enhanced β-casein promotor luciferase activity and β-casein RNA expression in the HC11 and HC11-luci cell line compared to vector control. Because the expression of conditionally active-Akt-1 blocked lactogenic differentiation whereas dominant negative-Akt1 enhanced lactogenic differentiation, it appears that Akt activity contributes to the regulation of lactogenic differentiation in HC11 cells.

The effect of PI-3-K activity on prolactin dependent Stat5 phosphorylation and trans-activation

Stat5 phosphorylation by a receptor-associated Jak kinase results in Tyr694 phosphorylation, and Tyr694 phosphorylation is necessary for Stat5 activity (Clevenger, 2004). Serine phosphorylation (Ser726/731) of both Stat5A and Stat5B increases during late pregnancy and lactation and in the presence of glucocorticoids maintains β-casein synthesis (Beuvink, 2000; Yamashita, 2001). As elucidated previously (Cerrito, 2004), blocking the Mek/Erk pathway with a DN-Ras enhanced Stat5 Tyr694 phosphorylation in response to prolactin. Therefore, the role of PI-3-K pathway on Stat5 Tyr694 and Ser726/731 phosphorylation was investigated. HC11-luci cells were grown to confluence and serum starved for 16 hours prior to prolactin stimulation. The cells were pre-treated with EGF or EGF and LY294002 and then stimulated with prolactin. Nuclear extracts were prepared from cells and total Stat5 was immunoprecipitated and analyzed by western blotting with antibodies for phospho-Stat5 or total Stat5. The addition of EGF reduced prolactin-induced Stat5 Tyr694 and Ser726/731 phosphorylation (Figure 4), and

PI-3-K inhibition moderately restored Stat5 phosphorylation in the presence of EGF as compared to the DIP treated cells (Figure 4).

In addition to β-casein and other milk proteins whose transcription requires Stat5 phosphorylation as well as dexamethasone-mediated events, a number of other genes are transcriptionally activated by prolactin (or DIP) stimulation but blocked by EGF. One of these genes, prolactin-induced protein (PIP), was transcriptionally activated by DIP and its transcription was blocked by EGF addition at the time of DIP administration. Northern blot analysis revealed that, unlike the case of β-casein, the addition of LY294002 did not prevent EGF from blocking PIP transcription (supplemental data) but inhibiting the Mek/Erk pathway with PD98059 blocked EGF dependent inhibition of PIP transcription. The incomplete ability of LY294002 to rescue the EGF-induced block on prolactin- or DIP- induced transcription suggests that some of the effects of the PI-3-kinase pathway are independent of Stat5 phosphorylation, DNA binding and transcriptional activation.

EGF activates p38 Kinase, JNK and p70S6 Kinase via PI-3-K and mTOR dependent mechanisms in HC11 mammary epithelial cells

Our previously published data as well as other reports suggest that many signal transduction pathways are stimulated by EGF in HC11 cells as well as various other cell types (Cerrito, 2004; Lee, 2002). p38MAPK has been identified as a potential downstream target of EGF signaling (Daly, 1999). In addition, Akt, which is stimulated by EGF, directly activates mTOR (Vivanco, 2002). The effect of other signal transduction inhibitors on lactogenic differentiation, alone or in combination, was determined in HC11 cells. SB20350, a p38 Kinase inhibitor, and Rapamycin, an mTOR inhibitor were added to HC11-luci cells in DIP-induction media in the presence or

absence of EGF. Luciferase activity was measured 48 hours post-induction and normalized to protein concentration. Rapamycin and SB203580 rescued the HC11-luci cells from EGF disruption of lactogenic differentiation demonstrating that EGF blocks differentiation in part via mTOR and p38 Kinase dependent pathways (Figure 5). Interestingly, however, the combination of the PI-3-K and Mek/Erk inhibitors increased β-casein promotor luciferase activity by 15 fold over the PI-3-K and Mek/Erk inhibitors alone. This result suggests that the PI-3-K and Mek/Erk signaling pathways are independent and synergistic in their activation by EGF.

The results above implicated PI-3-K and mTOR signaling as important pathways capable of blocking lactogenic differentiation. HC11 cells were examined to more fully characterize the effect of PI-3-K and mTOR chemical inhibitors on several signal transduction pathways induced by EGF. HC11 cells were serum starved in the absence of EGF and incubated 4 hours with LY294002 and Rapamycin prior to stimulation with EGF for times indicated. The cell lysates were harvested and analyzed by western blotting for expression and phosphorylation of the indicated proteins. The PI-3-K inhibitor completely blocked the phosphorylation and subsequent activation of Akt (Ser473) and p70S6K (Thr389) and partially blocked the phosphorylation and activation of p38 (Thr183/Tyr182) and SAPK/JNK (Thr183/Tyr185) (Figure 6). The mTOR inhibitor completely blocked the activation of p38, SAPK/JNK and p70S6K. However, neither inhibitor blocked the activation of Erk. The data demonstrates that EGF activation of p38, SAPK/JNK and p70S6K in HC11 cells is PI-3-K and mTOR dependent.

EGF stimulation phosphorylates Ribosomal Protein S6, elongation initiation factor 4E, eIF4E-binding protein 1 via PI-3-K/mTOR dependent mechanisms in HC11 mammary epithelial cells

One well recognized pathway involved in cell growth and proliferation via the regulation of protein synthesis is Akt/mTOR/p70S6K (Hay, 2004). Therefore, we investigated the activation state of proteins downstream of p70S6K in response to EGF in order to elucidate PI-3-K's potential role in HC11 cell protein synthesis. HC11 cells were grown to confluence in EGF containing media. The cells were serum starved in the absence of EGF and incubated with LY294002, Rapamycin and PD98059 prior to stimulation with EGF for the times indicated. Lysates were harvested and analyzed by western blotting using antibodies specific for phosphorylated and non-phosphorylated forms of the indicated proteins. The PI-3-K and mTOR inhibitors blocked the phosphorylation of elongation initiation factor 4E (eIF4E)(Ser289), eIF4E-binding protein 1 (4E-BP1) (Ser65), as well as ribosomal protein S6 (RPS6) (Ser235/236). The Mek/Erk inhibitor blocked the phosphorylation of MNK1 (Thr197/202), which is known to be Mek/Erk dependent (Parra, 2005) (Figure 7). In addition to the chemical inhibitors, expression of a conditionally active-Akt and was tested to assess p70S6K activation (Figure 8). The expression of conditionally active-Akt caused constitutive activation of p70S6K. Therefore the evidence suggests that one mechanism by which PI-3-K activation prevents lactogenic differentiation in HC11 mammary epithelial cells may involve the activation of p70S6K, and the subsequent phosphorylation of RPS6, eIF4E, and 4E-BP1.

The studies described above addressed short-term stimulation of cells with EGF. Next, the long-term activation of signal transduction pathways dependent on PI-3-K stimulation was examined in HC11 cells (Figure 9). HC11 cells were induced to differentiate in DIP-induction media with and without EGF for times up to 48 hours. LY294002 was added at time of induction. Lysates were analyzed by western blotting for phosphorylation of specific proteins. In the HC11 cells stimulated with DIP the activation of p70S6K (Thr389) completely diminished by approximately 12 hours, whereas the cells stimulated with DIP in the presence of EGF the activation of p70S6K was persistent through 24 hours. In contrast, the cells treated with LY294002 showed no p70S6K activation at any time point after induction. These results suggest that blocking the PI-3-K pathway at the time of DIP-induction enhanced differentiation via a similar mechanism to that described above, *i.e.* inactivation of Akt/mTOR/p70S6K.

Discussion

Carcinogenesis is usually a multi-step process involving aberrant regulation of multiple cellular processes and/or signaling pathways. In becoming cancerous, a cell generally devises methods for averting a numerous molecular, cellular, and systemic protective mechanisms (Hahn, 2002). Molecular dissection of the cellular pathways implicated in tumorigenesis is, therefore, a high priority in cancer research.

Mammary epithelial cells undergo episodic cycles of growth, differentiation and apoptosis throughout a female's life. Mammary gland development can be divided into seven stages: embryonic, postnatal, juvenile, puberty, pregnancy, lactation, and involution. One of the leading risk factors for breast cancer is nullparity (Simpson, 2002). Without the induction of lactogenesis (differentiation) followed by involution (apoptosis) a woman is more likely to develop breast cancer. Hence, the delineation of factors that inhibit lactogenesis is crucial to our understanding of breast cancer.

Up to 30% of all breast tumors over-express one or more members of the epidermal growth factor receptor family (ErbB), and these molecules are common targets for breast cancer therapy (LeVea, 2004; Reise, 1998; Danielsen, 2003). Excess activation of signaling pathways downstream of the epidermal growth factor receptor has been directly linked to breast cancer development and chemotherapeutic resistance (Navolanic, 2003). While epidermal growth factor is required for normal mammary epithelial cell proliferation, it has been shown to inhibit lactogenic differentiation of mammary epithelial cells both *in vitro* and *in vivo* (Hynes, 1990; Brandt, 2001; Cerrito, 2004). The two main signaling pathways activated by EGF stimulation in HC11 mammary epithelial cells are the Ras/Mek/Erk and the PI-3-K pathways (De Santis, 1997).

The phosphatidylinositol-3-kinase (PI-3-K) pathway is important in tumorigenesis in several ways. Aberrant PI-3-K activation has been demonstrated to promote both proliferation and survival of transformed cells, including those exhibiting EGF-dependent transformation (Lowe, 2004; Fry, 2001). The deregulation of many PI-3-K pathway components has recently been linked to a number of human malignancies (Vivanco, 2002; Fresno, 2004); elevated Akt levels, for example, have been found in breast, ovarian, colon and thyroid cancers (Lou, 2003; Vivanco, 2002). Comparisons of PI-3-K expression levels in tumor tissue from pre-menopausal versus post-menopausal patients demonstrated that the pre-menopausal tumors, noted for their associated poor prognosis and chemoresistance, expressed significantly higher levels of PI-3-K (Gershtein, 1999). The Yu laboratory demonstrated that the phosphorylation of Akt/mTOR increased progressively from normal breast epithelia to hyperplasia and abnormal hyperplasia to tumor invasion, suggesting that PI-3-K activity levels directly correlate with the degree of tumor progression (Zhou, 2004). These findings not only demonstrate the prominence of PI-3-K expression in breast cancer and its utility as prognostic indicator, but also lead to the speculation that ligand-induced and/or constitutive PI-3-K pathway activation might represent an important step in breast tumorigenesis.

Therefore, the present study investigates the effects of EGF on the PI-3-K pathway in HC11 mammary epithelial cells and its contribution in blocking lactogenic hormone induced differentiation. Our first set of data confirmed our earlier results indicating that the addition of the PI-3-K inhibitor LY294002 rescued the EGF block of β-casein luciferase activity, β-casein transcription and mammosphere formation. This suggests that hormone induced lactogenic differentiation is, in part, dependent on PI-3-K activity.

Furthermore, the expression of a conditionally active-Akt-1 blocked lactogenic differentiation whereas dominant negative-Akt1 enhanced it. These results indicate that EGF blocks HC11 lactogenic differentiation in part via a PI-3-K/Akt dependent mechanism. β-casein transcription requires dexamethasone, insulin, and prolactin in HC11 cells (Merlo, 1996). Prolactin phosphorylates Stat5A/B which then translocates to the nucleus where it binds to the β-casein promotor and activates its transcription. Therefore, the phosphorylation of Stat5 is one measure of the initiation of lactogenic differentiation. PI-3-K inhibition in HC11 cells partially prevented the EGF block of Stat5 Tyr and Ser phosphorylation. This agrees with our previous findings that demonstrated an increase in Stat5 DNA binding following PI-3-K inhibition (Cerrito, 2004). PI-3-K inhibition does not rescue the transcription of all prolactin-induced targets that are blocked by EGF in mammary epithelial cells. For example, EGF blocks PIP transcription via the Mek/Erk, not PI-3-K pathways (supplemental data). Consequently, we conclude that the involvement of the PI-3-K pathway in blocking lactogenic differentiation is partly independent of Stat5-induced transcription. We hypothesize that the inhibitory effect of PI-3-K on β-casein transcription and β-casein promotor luciferase activity is likely through combined prolactin and dexamethasone-mediated mechanisms.

Through the use of chemical inhibitors alone or in combination our data revealed that the PI-3-K and Mek/Erk signaling pathways are independent and synergistic in their block of HC11 lactogenic differentiation. We determined that EGF activates Akt, mTOR, p70S6 Kinase, Ribosomal protein S6, eIF4E and 4E-BP1 in a PI-3-K dependent manner. Evidence therefore suggests that one mechanism by which PI-3-K activation prevents lactogenic differentiation in HC11 mammary epithelial cells is by regulating the

synthesis of proteins involved in this process. In addition, our data indicate that PI-3-K regulated the subsequent activation of GSK3β (supplemental data). One recent report shows that cyclin D1 expression in a breast cancer cell line, T47D, is dependent on PI-3-K activation (Neve, 2002). We verify that cyclin D1 transcription and expression are dependent on PI-3-K activity in HC11 mammary epithelial cells. Previous data suggested that the regulation of osteopontin (OPN), (Zhang, 2003), which has been strongly correlated with tumor metastasis, is PI-3-K dependent. Our studies show that OPN is transcriptionally regulated by PI-3-K in HC11 cells (supplemental data). Thus, we conclude that PI-3-K regulates the transcription of genes important in tumorigenesis.

The present study enhances our knowledge of normal mammary epithelial differentiation in several ways. We confirm that PI-3-K inhibits hormone induced lactogenic differentiation in mammary epithelial cells (Merlo, 1996; De Santis, 1997). Two previous studies questioned whether PI-3-K activation of Akt in normal mammary epithelial cells is sufficient for cellular transformation (Zeng, 2002; Xie, 2003). We demonstrated that PI-3-K activates Akt following EGF stimulation in HC11 cells. Moreover, conditionally activated-Akt-1 blocks lactogenic differentiation and dominant negative-Akt1 rescued HC11 cells from an EGF-induced inhibition of differentiation. This confirms that Akt plays a significant role in the PI-3-K pathways in HC11 cells. Several studies have also suggested that the activation of Erk is regulated through the PI-3-K pathway (Mahimainathan, 2005; Qiao, 2004), but our data demonstrated that EGF stimulation of Erk activation in HC11 mammary epithelial cells was not altered by LY294002. In addition, our previous work revealed that PI-3-K is activated directly by EGF and not by Ras (Cerrito, 2004). While no studies have addressed the mechanism

by which PI-3-K blocks lactogenic differentiation, we demonstrated that the inhibition of PI-3-K, Akt or mTOR blocked the activation p70S6 Kinase and its downstream targets. Interestingly, we discovered that PDK1 is constitutively phosphorylated in HC11 cells and this is not blocked by LY294002. We also demonstrated that the expression of a conditionally active-Akt leads to the constitutive activation of p70S6 Kinase. PDK1 has been shown to directly activate p70S6 Kinase independent of Akt, but our results suggest that the activation of p70S6 Kinase is dependent on Akt and mTOR in HC11 cells (Pullen, 1998).

In conclusion, we present evidence that suggests that PI-3-K affects the synthesis of proteins involved in blocking differentiation as well as genes that contribute to cell cycle progression and tumorigenesis. Our study also demonstrates, for the first time, a comprehensive investigation of the role in which PI-3-K plays in hormone induced HC11 lactogenic differentiation. Many studies have addressed this pathway in cancer models (Gershtein, 1999; Yu, 2004; Ripple, 2005; Koziczak, 2004) however, never fully elucidating the pathway during normal mammary differentiation. Since epidemiological studies report that the leading risk for developing breast cancer is nullparity and lactogenesis and involution are the key protective events, our study addresses the importance of understanding the signaling mechanisms that inhibit this process. Hence, the understanding of normal signaling during lactogenic differentiation is important to our understanding of breast tumors that over-express PI-3-K. This understanding may ultimately lead to the development of breast cancer prevention techniques as well as therapeutic approaches in treating tumors where excessive PI-3-K activity is detected.

References

Bailey, J. P., K. M. Nieport, et al. (2004). "Prolactin and transforming growth factor-beta signaling exert opposing effects on mammary gland morphogenesis, involution, and the Akt-forkhead pathway." Mol Endocrinol 18(5): 1171-84.

Ball, R. K., R. R. Friis, et al. (1988). "Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line." Embo J 7(7): 2089-95.

Beuvink, I., D. Hess, et al. (2000). "Stat5a serine phosphorylation. Serine 779 is constitutively phosphorylated in the mammary gland, and serine 725 phosphorylation influences prolactin-stimulated in vitro DNA binding activity." J Biol Chem 275(14): 10247-55.

Blatchford, D. R., L. H. Quarrie, et al. (1999). "Influence of microenvironment on mammary epithelial cell survival in primary culture." J Cell Physiol 181(2): 304-11.

Brandt, R., A. M. Wong, et al. (2001). "Mammary glands reconstituted with Neu/ErbB2 transformed HC11 cells provide a novel orthotopic tumor model for testing anti-cancer agents." Oncogene 20(39): 5459-65.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science 296(5573): 1655-7.

Carpenter, C. L. and L. C. Cantley (1996). "Phosphoinositide 3-kinase and the regulation of cell growth." Biochim Biophys Acta 1288(1): M11-6.

Cerrito, M. G., T. Galbaugh, et al. (2004). "Dominant negative Ras enhances lactogenic hormone-induced differentiation by blocking activation of the Raf-Mek-Erk signal transduction pathway." J Cell Physiol 201(2): 244-58.

Daly, J. M., M. A. Olayioye, et al. (1999). "NDF/heregulin-induced cell cycle changes and apoptosis in breast tumour cells: role of PI3 kinase and p38 MAP kinase pathways." Oncogene 18(23): 3440-51.

Daly, R. J., H. Gu, et al. (2002). "The docking protein Gab2 is overexpressed and estrogen regulated in human breast cancer." Oncogene 21(33): 5175-81.

Danielsen, A. J. and N. J. Maihle (2002). "The EGF/ErbB receptor family and apoptosis." Growth Factors 20(1): 1-15.

De Santis, M. L., S. Kannan, et al. (1997). "Cripto-1 inhibits beta-casein expression in mammary epithelial cells through a p21ras-and phosphatidylinositol 3'-kinase-dependent pathway." Cell Growth Differ 8(12): 1257-66.

Ebert, A. D., C. Wechselberger, et al. (1999). "Cripto-1 induces phosphatidylinositol 3'-kinase-dependent phosphorylation of AKT and glycogen synthase kinase 3beta in human cervical carcinoma cells." Cancer Res 59(18): 4502-5.

Fingar, D. C., S. Salama, et al. (2002). "Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E." Genes Dev 16(12): 1472-87.

Fresno Vara, J. A., E. Casado, et al. (2004). "PI3K/Akt signalling pathway and cancer." Cancer Treat Rev 30(2): 193-204.

Fruman, D. A., R. E. Meyers, et al. (1998). "Phosphoinositide kinases." Annu Rev Biochem 67: 481-507.

Fry, M. J. (2001). "Phosphoinositide 3-kinase signalling in breast cancer: how big a role might it play?" Breast Cancer Res 3(5): 304-12.

Fujio, Y. and K. Walsh (1999). "Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner." J Biol Chem 274(23): 16349-54.
Gershtein, E. S., V. A. Shatskaya, et al. (1999). "Phospatidylinositol 3-kinase expression in human breast cancer." Clin Chim Acta 287(1-2): 59-67.
Hahn, W. C. and R. A. Weinberg (2002). "Modelling the molecular circuitry of cancer." Nat Rev Cancer 2(5): 331-41.
Hay, N. and N. Sonenberg (2004). "Upstream and downstream of mTOR." Genes Dev 18(16): 1926-45.
Holz, M. K. and J. Blenis (2005). "Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase." J Biol Chem 280(28): 26089-93.
Horsch, K., M. D. Schaller, et al. (2001). "The protein tyrosine phosphatase-PEST is implicated in the negative regulation of epidermal growth factor on PRL signaling in mammary epithelial cells." Mol Endocrinol 15(12): 2182-96.
Humphreys, R. C. and J. M. Rosen (1997). "Stably transfected HC11 cells provide an in vitro and in vivo model system for studying Wnt gene function." Cell Growth Differ 8(8): 839-49.
Hynes, N. E., D. Taverna, et al. (1990). "Epidermal growth factor receptor, but not c-erbB-2, activation prevents lactogenic hormone induction of the beta-casein gene in mouse mammary epithelial cells." Mol Cell Biol 10(8): 4027-34.
Kohn, A. D., A. Barthel, et al. (1998). "Construction and characterization of a conditionally active version of the serine/threonine kinase Akt." J Biol Chem 273(19): 11937-43.
Koziczak, M., T. Holbro, et al. (2004). "Blocking of FGFR signaling inhibits breast cancer cell proliferation through downregulation of D-type cyclins." Oncogene 23(20): 3501-8.
Lee, J. T., Jr. and J. A. McCubrey (2002). "The Raf/MEK/ERK signal transduction cascade as a target for chemotherapeutic intervention in leukemia." Leukemia 16(4): 486-507.
LeVea, C. M., J. E. Reeder, et al. (2004). "EGF-dependent cell cycle progression is controlled by density-dependent regulation of Akt activation." Exp Cell Res 297(1): 272-84.
Luo, J., B. D. Manning, et al. (2003). "Targeting the PI3K-Akt pathway in human cancer: rationale and promise." Cancer Cell 4(4): 257-62.
Mahimainathan, L., N. Ghosh-Choudhury, et al. (2005). "EGF stimulates mesangial cell mitogenesis via PI3-kinase-mediated MAPK-dependent and AKT kinase-independent manner: involvement of c-fos and p27Kip1." Am J Physiol Renal Physiol 289(1): F72-82.
Marte, B. M., T. Meyer, et al. (1994). "Protein kinase C and mammary cell differentiation: involvement of protein kinase C alpha in the induction of beta-casein expression." Cell Growth Differ 5(3): 239-47.
Merlo, G. R., D. Graus-Porta, et al. (1996). "Growth, differentiation and survival of HC11 mammary epithelial cells: diverse effects of receptor tyrosine kinase-activating peptide growth factors." Eur J Cell Biol 70(2): 97-105.

Navolanic, P. M., L. S. Steelman, et al. (2003). "EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review)." Int J Oncol 22(2): 237-52.
Neve, R. M., T. Holbro, et al. (2002). "Distinct roles for phosphoinositide 3-kinase, mitogen-activated protein kinase and p38 MAPK in mediating cell cycle progression of breast cancer cells." Oncogene 21(29): 4567-76.
Parra, J. L., M. Buxade, et al. (2005). "Features of the catalytic domains and C termini of the MAPK signal-integrating kinases Mnk1 and Mnk2 determine their differing activities and regulatory properties." J Biol Chem 280(45): 37623-33.
Petersen, H. and L. A. Haldosen (1998). "EGF modulates expression of STAT5 in mammary epithelial cells." Exp Cell Res 243(2): 347-58.
Pullen, N., P. B. Dennis, et al. (1998). "Phosphorylation and activation of p70s6k by PDK1." Science 279(5351): 707-10.
Qiao, M., P. Shapiro, et al. (2004). "Insulin-like growth factor-1 regulates endogenous RUNX2 activity in endothelial cells through a phosphatidylinositol 3-kinase/ERK-dependent and Akt-independent signaling pathway." J Biol Chem 279(41): 42709-18.
Riese, D. J., 2nd and D. F. Stern (1998). "Specificity within the EGF family/ErbB receptor family signaling network." Bioessays 20(1): 41-8.
Ripple, M. O., S. Kalmadi, et al. (2005). "Inhibition of either phosphatidylinositol 3-kinase/Akt or the mitogen/extracellular-regulated kinase, MEK/ERK, signaling pathways suppress growth of breast cancer cell lines, but MEK/ERK signaling is critical for cell survival." Breast Cancer Res Treat 93(2): 177-88.
Shen, Q. and P. H. Brown (2003). "Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways." J Mammary Gland Biol Neoplasia 8(1): 45-73.
Simpson, H. W., C. S. McArdle, et al. (2002). "Pregnancy postponement and childlessness leads to chronic hypervascularity of the breasts and cancer risk." Br J Cancer 87(11): 1246-52.
Taverna, D., B. Groner, et al. (1991). "Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-erbB-2 receptor activation all promote growth but have distinctive effects upon mouse mammary epithelial cell differentiation." Cell Growth Differ 2(3): 145-54.
Tonko-Geymayer, S., O. Goupille, et al. (2002). "Regulation and function of the cytokine-inducible SH-2 domain proteins, CIS and SOCS3, in mammary epithelial cells." Mol Endocrinol 16(7): 1680-95.
Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." Nat Rev Cancer 2(7): 489-501.
Wartmann, M., N. Cella, et al. (1996). "Lactogenic hormone activation of Stat5 and transcription of the beta-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity." J Biol Chem 271(50): 31863-8.
Wendel, H. G., E. De Stanchina, et al. (2004). "Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy." Nature 428(6980): 332-7.

Wyszomierski, S. L., J. Yeh, et al. (1999). "Glucocorticoid receptor/signal transducer and activator of transcription 5 (STAT5) interactions enhance STAT5 activation by prolonging STAT5 DNA binding and tyrosine phosphorylation." Mol Endocrinol 13(2): 330-43.

Xie, J., M. J. LeBaron, et al. (2002). "Role of tyrosine kinase Jak2 in prolactin-induced differentiation and growth of mammary epithelial cells." J Biol Chem 277(16): 14020-30.

Xie, Z., X. Zeng, et al. (2003). "Transformation of mammary epithelial cells by 3-phosphoinositide- dependent protein kinase-1 activates beta-catenin and c-Myc, and down-regulates caveolin-1." Cancer Res 63(17): 5370-5.

Yamashita, H., M. T. Nevalainen, et al. (2001). "Role of serine phosphorylation of Stat5a in prolactin-stimulated beta-casein gene expression." Mol Cell Endocrinol 183(1-2): 151-63.

Zeng, X., H. Xu, et al. (2002). "Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with the induction of protein kinase Calpha." Cancer Res 62(12): 3538-43.

Zhang, G., B. He, et al. (2003). "Growth factor signaling induces metastasis genes in transformed cells: molecular connection between Akt kinase and osteopontin in breast cancer." Mol Cell Biol 23(18): 6507-19.

Zhou, X., M. Tan, et al. (2004). "Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers." Clin Cancer Res 10(20): 6779-88.

Figure Legends

Figure 1. The effect of the chemical inhibitor of PI-3-K, LY294002 on epidermal growth factor (EGF) disruption of differentiation **A**: HC11 cells containing a luciferase gene under the control of a β-casein promotor (HC11-luci) were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media in the presence or absence of EGF (10ng/ml). Inhibitors were added at the time of induction at previously determined optimal concentrations (PD98059 20μM, LY294002 10μM, and wortmannin 100nM,) and activity was determined 48 hours post-induction. Luciferase activity was normalized to protein. *These values represent statistically significant difference (*P* value .0001) from the DIP+EGF. **B**: HC11 cells were grown to confluence as stated above and induced to differentiate in DIP-induction media with and without EGF (10ng/ml). Inhibitors were added at time of induction at slightly lower concentrations to avoid toxicity (LY294002 5μM). Total cell RNA was harvested at 48 hours after induction. β-casein induction was determined via northern blot. For quantitation β-casein expression at 48 hours was normalized to β-Actin. **C**: HC11 cells were grown to confluence as stated above and induced to differentiate in DIP-induction media with and without EGF (10ng/ml). LY294002 (10μM) was added at time of induction. Cells were photographed at 96 hours post-induction. The number of mammospheres per field is reported: this was determined by counting the number of mammospheres per low power field and determining the mean of five fields.

Figure 2. The effect of conditionally active-Akt-1 on EGF disruption of differentiation. The HC11-luci cells were grown to 80% confluence and transiently transfected with either a conditionally active-Akt-1 (CA-Akt) (myrΔ4-129-ER) or a control vector (pCDNA3.1). The following day the cells were incubated in DIP-induction media plus tamoxifen (activates the conditionally active-Akt-1). **A:** Luciferase activity was determined 48 hours post-induction. Luciferase activity was normalized to protein. **B:** Expression of Akt was shown via western blot. Gel was loaded with equal amounts of protein (117.5μg).

Figure 3. The effect of dominant negative-Akt1 (DN-Akt) adenoviral infection on EGF disruption of differentiation. **A**: HC11-luci cells were grown to 90% confluence, infected with either a DN-Akt1 or control (LacZ) adenovirus. Cells were changed to DIP-induction media the next day, and cells were harvested 48 hours post-induction for β-casein promotor luciferase activity. **B**: HC11 cells were grown and treated as stated above. Cells were harvested 48 hours post-induction for β-casein RNA expression by northern blot. Expression of Akt was shown in both cell lines via western blot analysis and gels were loaded with equal amount of protein (120μg).

Figure 4. The effect of LY294002 on Stat5 phosphorylation. HC11-luci cells were grown to confluence and serum starved over night prior to prolactin (PRL) stimulation. The cells were pre-treated with EGF or EGF and LY294002 (10μM) for four hours and then stimulated with PRL (5 μg/ml) and nuclear extracts were prepared from cells at 0, 15 and 30 min. Total Stat5 was immuno-precipitated and analyzed by western blotting

with antibodies for phospho-Stat5 (P-Stat5) or total Stat5 (T-Stat5). The amount of phospho-Stat5 and total Stat5 on the western blots was quantitated, and the amount of phospho-Stat5 was normalized to the total Stat5 and reported in relative units.

Figure 5. The effect of signal transduction inhibitors on epidermal growth factor (EGF) disruption of differentiation. HC11-luci cells were grown to confluence in EGF-containing media then induced to differentiate in DIP-induction media in the presence or absence of EGF (10ng/ml). Inhibitors were added alone or in combination at the time of induction (LY294002 10μM, SB203580 10μM, Rapamycin 100nM, PD98059 20μM). Luciferase activity was determined 48 hours post-induction and normalized to protein concentration. *These values represent statistically significant difference (*P* value .0001) from the DIP+EGF.

Figure 6. The effect of chemical inhibitors on signal transduction pathways on HC11 cells. HC11 cells were grown to confluence in EGF-containing media. The cells were incubated in media without EGF and serum overnight and incubated four hours with LY294002 (LY) (10μM) and Rapamycin (Rap) (50nM) prior to re-stimulation with EGF (100ng/ml) for times indicated. Lysates were harvested and analyzed by western blotting using antibodies specific for phosphorylated and non-phosphorylated forms of the indicated proteins.

Figure 7. The effect of chemical inhibitors on the translational signal transduction pathway in HC11 cells. HC11 cells were grown to confluence in EGF-containing media. The cells were incubated in media without EGF and serum overnight and incubated four hours with **A:** LY294002 (LY)(10μM) and PD98059 (PD) (20μM) or **B:** LY294002 (LY) (10μM) and Rapamycin (Rap) (50nM) prior to re-stimulation with EGF (100ng/ml) for times indicated. Lysates were harvested and analyzed by western blotting using antibodies specific for phosphorylated and non-phosphorylated forms of the indicated proteins.

Figure 8. The effect of conditionally activated-Akt-1 on the translational signal transduction pathway in HC11 cells. The HC11 cells were grown to 80% confluence and transiently transfected with either a conditionally active-Akt (CA-Akt) (myrΔ4-129-ER) or a control vector (pCDNA3.1) HC11 cells. The following day the cells were incubated in media without EGF and serum overnight. The next day the cells were re-stimulation with EGF (100ng/ml) for times indicated. Lysates were harvested and analyzed by western blotting using antibodies specific for phosphorylated and non-phosphorylated forms of the indicated proteins.

Figure 9. The long-term activation of the translational signal transduction pathway in HC11 cells. HC11 cells were grown to confluence and induced to differentiate in DIP-induction media with and without EGF for times up to 48 hours. Lysates were harvested and analyzed by western blotting using antibodies specific for phosphorylated and non-phosphorylated forms of the indicated proteins.

Supplemental data. The effect of chemical inhibitors on cyclin D1 expression and GSK3β activation. HC11 cells were grown to confluence and induced to differentiate in DIP-induction media in the absence or presence of EGF (10ng/ml). The cells were treated with inhibitors at the time of induction at previously determined optimal concentrations (LY294002 10μM, Rapamycin 100nM, PD98059 20μM). Lysates were harvested and analyzed by western blotting using antibodies specific for phosphorylated and non-phosphorylated forms of the indicated proteins. The amount of cyclin D1 and phospho-GSK3β on the western blots was quantitated. Asterisk (*) and arrow denotes a reduction.

Supplemental data: The effect of signal transduction inhibitors on the EGF-dependent transcription of OPN and PIP in HC11 cells. The HC11 cells were induced to differentiate in DIP-induction media with and without EGF (10ng/ml). Inhibitors were added at time of induction alone (LY294002 10μM & 25μM, SB203580 10μM, Rapamycin 100nM, PD98059 20μM). Total cell RNA was harvested at 48 and 72 hours after transfer to DIP-induction media. PIP and OPN induction was determined via northern blot. For quantitation PIP and OPN transcription at 48 hours was normalized to β-Actin. *Denotes a reduction.

Figure 1

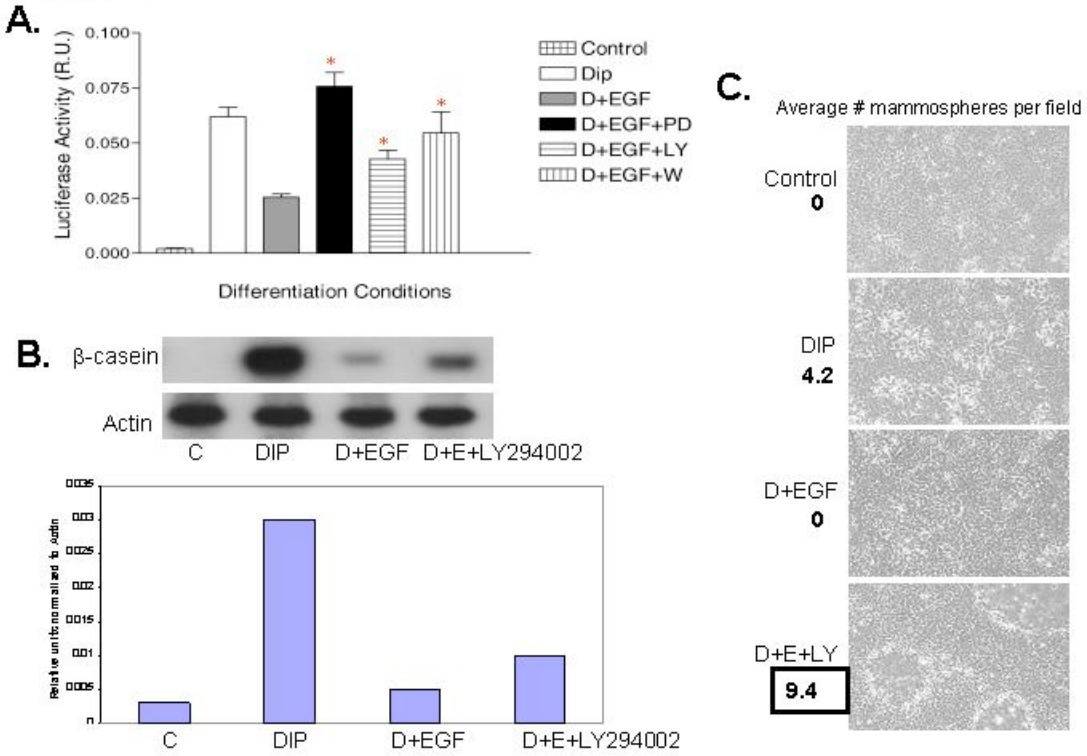

A.
Luciferase Activity (R.U.)
0.100
0.075
0.050
0.025
0.000
Control
Dip
D+EGF
D+EGF+PD
D+EGF+LY
D+EGF+W
Differentiation Conditions
B.
β-casein
Actin
C
DIP
D+EGF
D+E+LY294002
C
DIP
D+EGF
D+E+LY294002
C.
Average # mammospheres per field
Control
0
DIP
4.2
D+EGF
0
D+E+LY
9.4

Figure 2

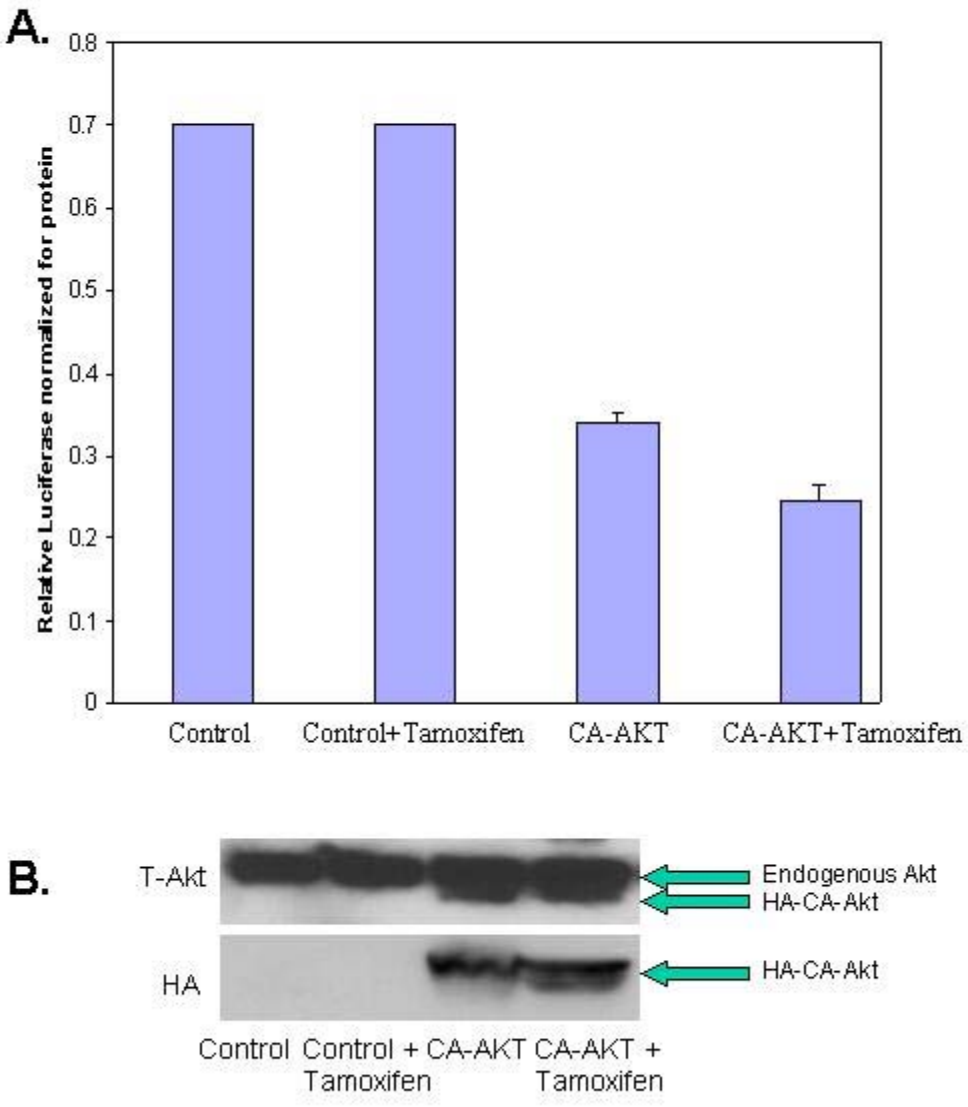

A.
Relative Luciferase normalized for protein
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Control+Tamoxifen
CA-AKT
CA-AKT+Tamoxifen
B.
T-Akt
HA
Endogenous Akt
HA-CA-Akt
HA-CA-Akt
Control
Control + Tamoxifen
CA-AKT
CA-AKT + Tamoxifen

Figure 3

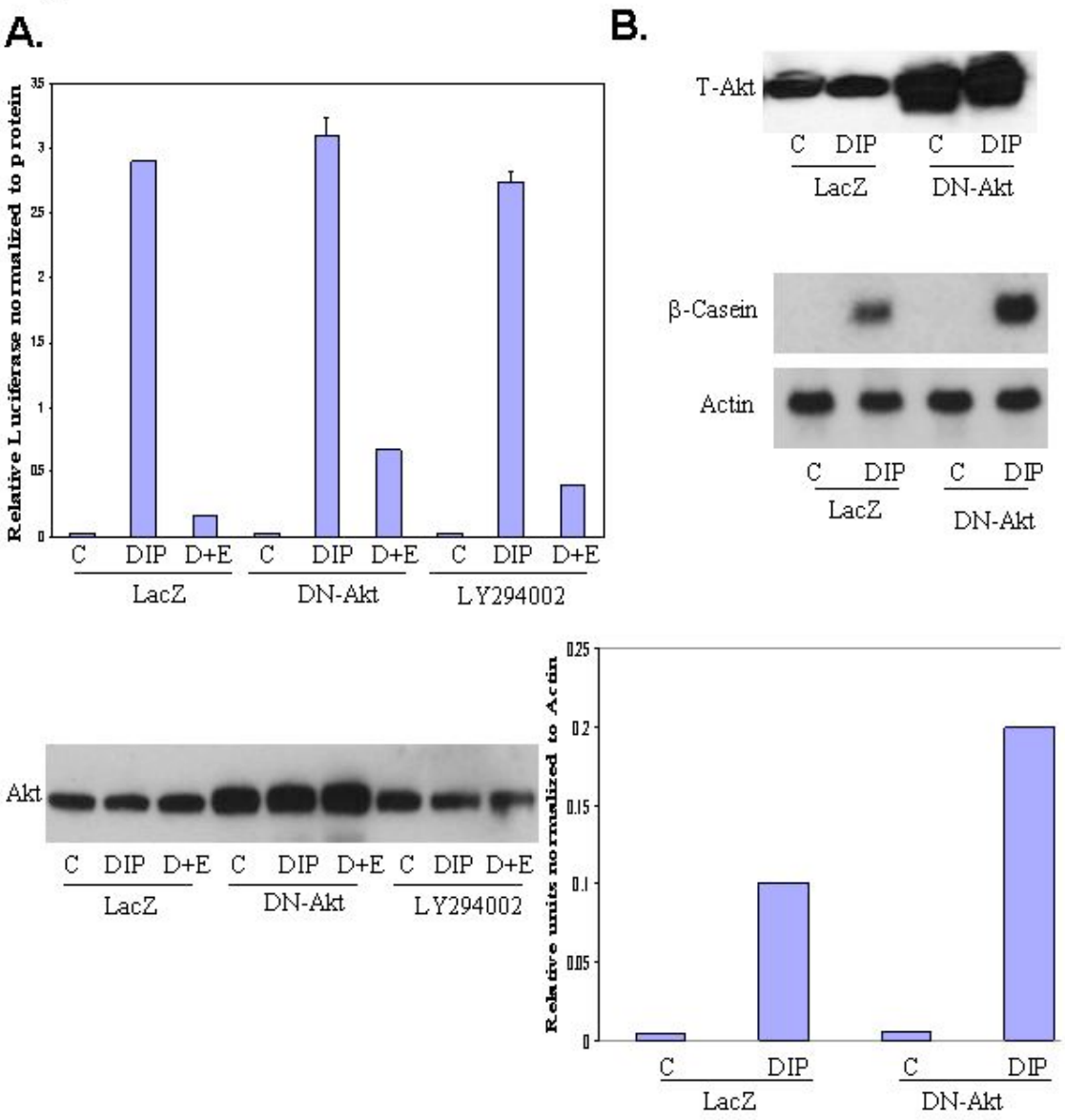

A.
B.
Relative Luciferase normalized to protein
C DIP D+E C DIP D+E C DIP D+E
LacZ
DN-Akt
LY294002
T-Akt
C DIP C DIP
LacZ
DN-Akt
β-Casein
Actin
C DIP C DIP
LacZ
DN-Akt
Akt
C DIP D+E C DIP D+E C DIP D+E
LacZ
DN-Akt
LY294002
Relative units normalized to Actin
C DIP C DIP
LacZ
DN-Akt

Figure 4

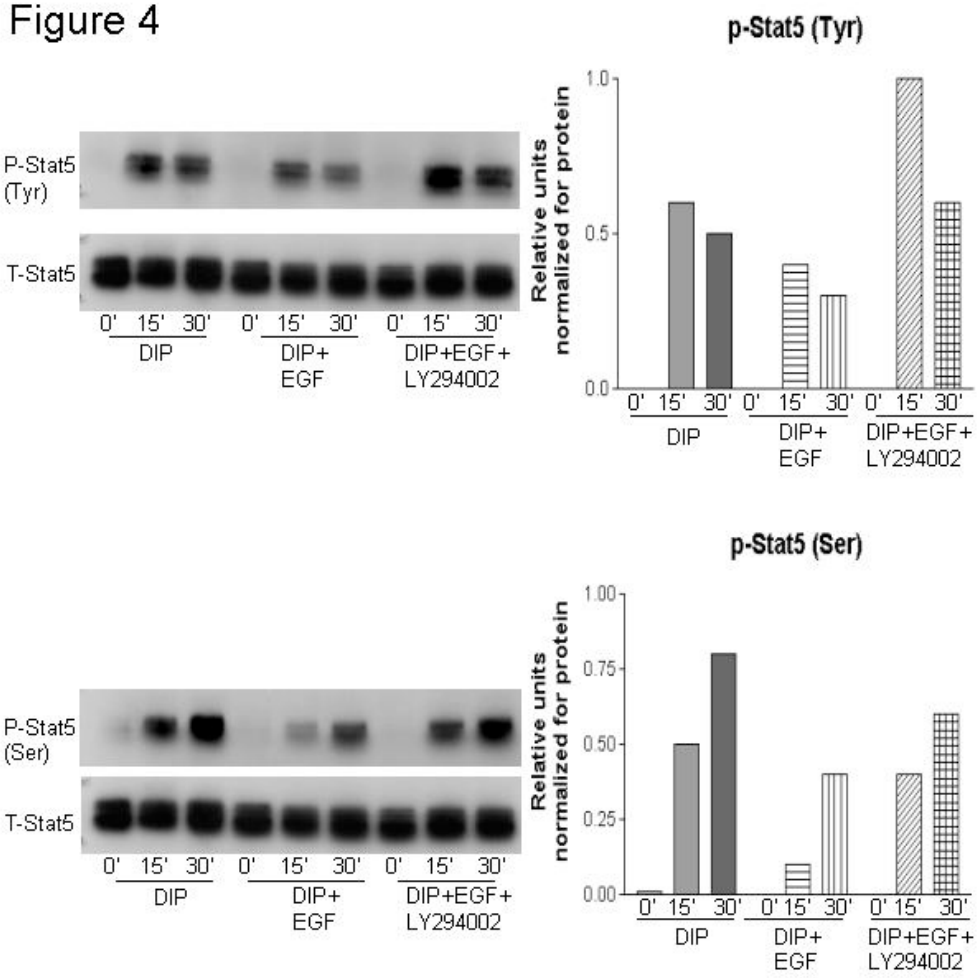

P-Stat5 (Tyr)
T-Stat5
0' 15' 30' 0' 15' 30' 0' 15' 30'
DIP
DIP+ EGF
DIP+EGF+ LY294002
p-Stat5 (Tyr)
Relative units normalized for protein
1.0
0.5
0.0
P-Stat5 (Ser)
T-Stat5
p-Stat5 (Ser)
1.00
0.75
0.50
0.25
0.00

Figure 5

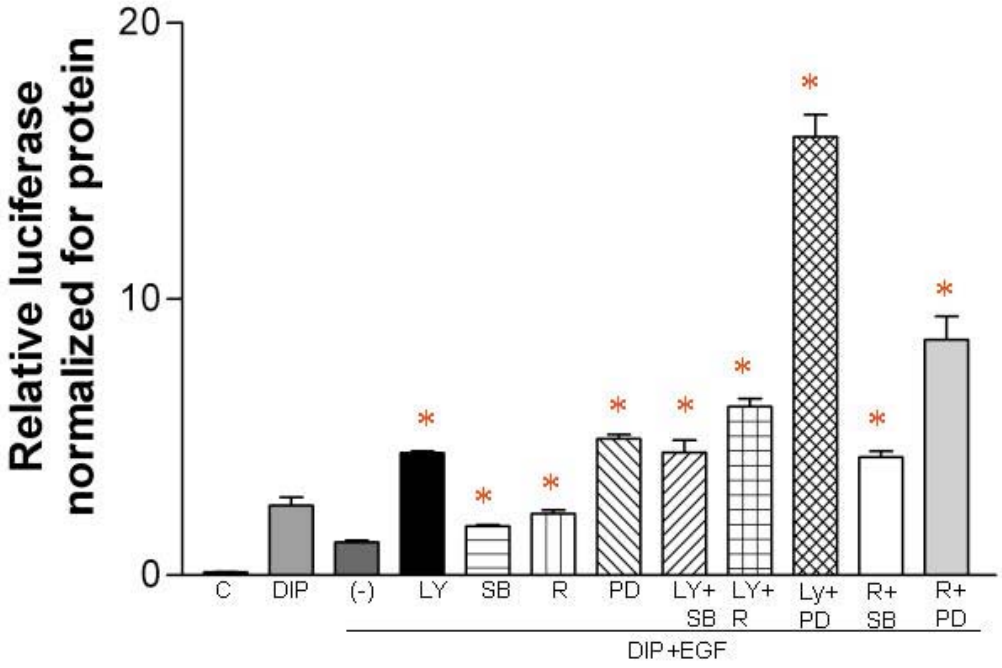

Relative luciferase normalized for protein
20
10
0
C
DIP
(-)
LY
SB
R
PD
LY+ SB
LY+ R
Ly+ PD
R+ SB
R+ PD
DIP+EGF

Figure 6

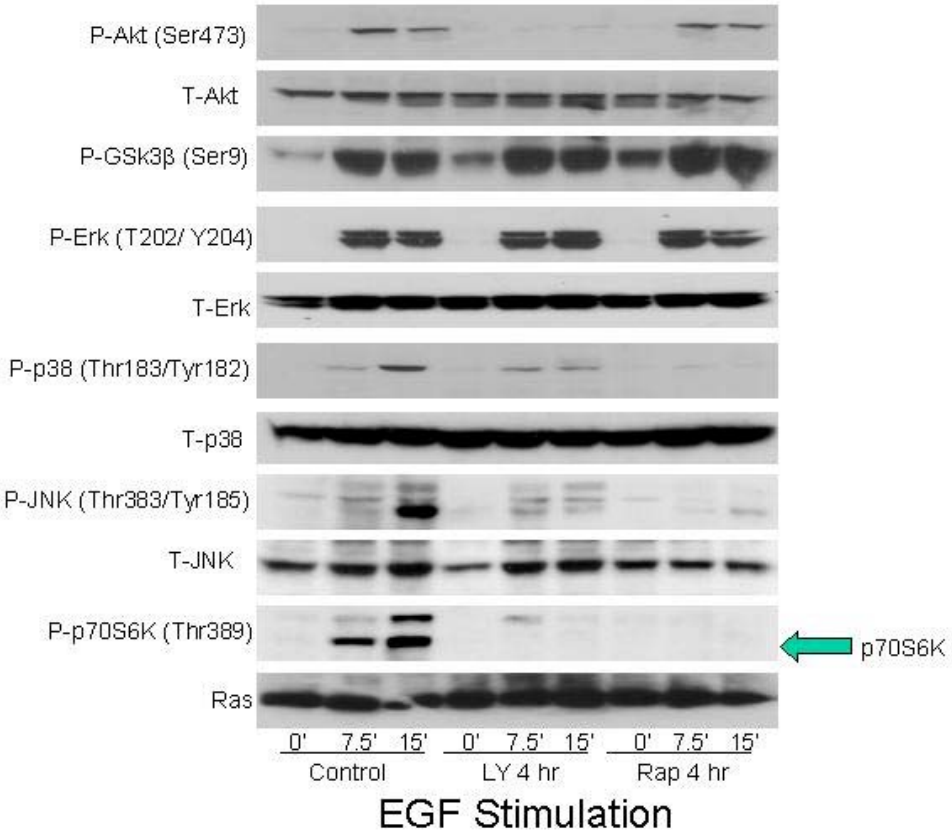
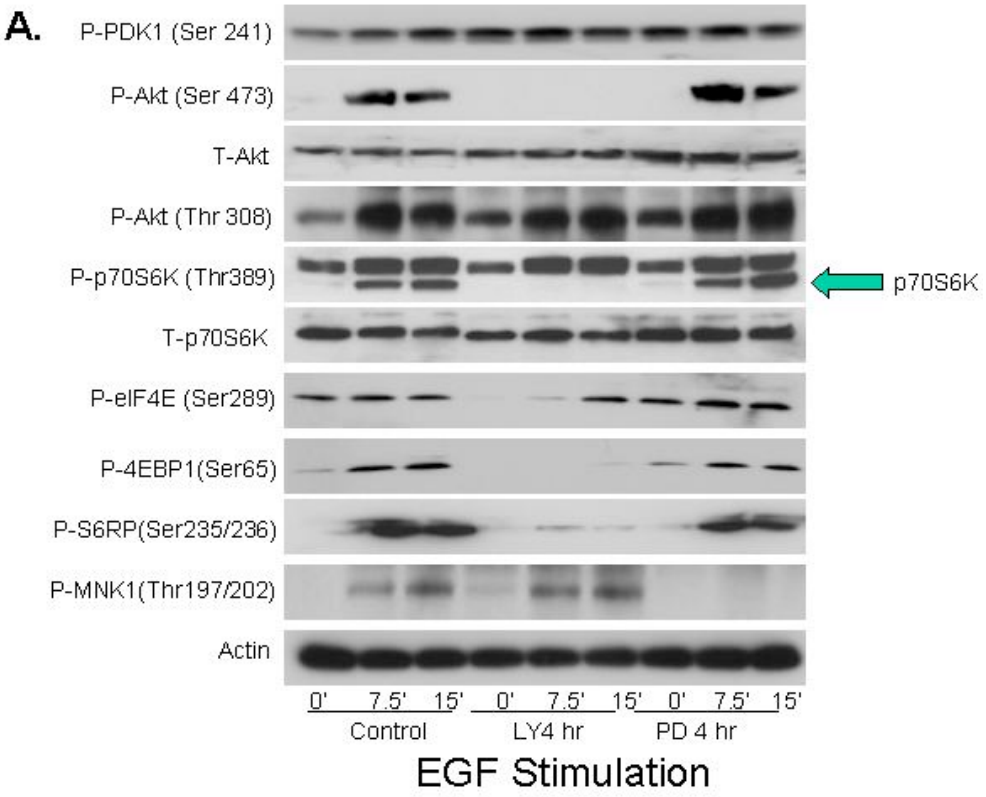

P-Akt (Ser473)
T-Akt
P-GSk3β (Ser9)
P-Erk (T202/ Y204)
T-Erk
P-p38 (Thr183/Tyr182)
T-p38
P-JNK (Thr383/Tyr185)
T-JNK
P-p70S6K (Thr389)
p70S6K
Ras
0' 7.5' 15' 0' 7.5' 15' 0' 7.5' 15'
Control
LY 4 hr
Rap 4 hr
EGF Stimulation

Figure 7


A.
P-PDK1 (Ser 241)
P-Akt (Ser 473)
T-Akt
P-Akt (Thr 308)
P-p70S6K (Thr389)
p70S6K
T-p70S6K
P-eIF4E (Ser289)
P-4EBP1(Ser65)
P-S6RP(Ser235/236)
P-MNK1(Thr197/202)
Actin
0' 7.5' 15' 0' 7.5' 15' 0' 7.5' 15'
Control
LY4 hr
PD 4 hr
EGF Stimulation

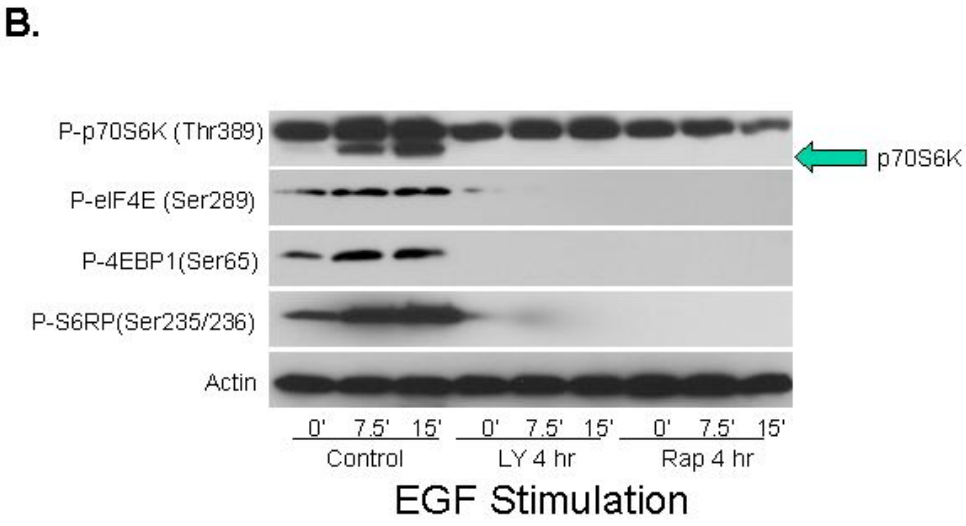

B.
P-p70S6K (Thr389)
p70S6K
P-eIF4E (Ser289)
P-4EBP1(Ser65)
P-S6RP(Ser235/236)
Actin
0' 7.5' 15' 0' 7.5' 15' 0' 7.5' 15'
Control
LY 4 hr
Rap 4 hr
EGF Stimulation

Figure 8

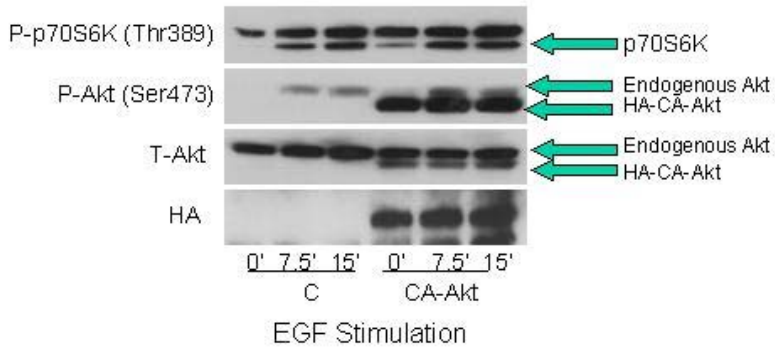
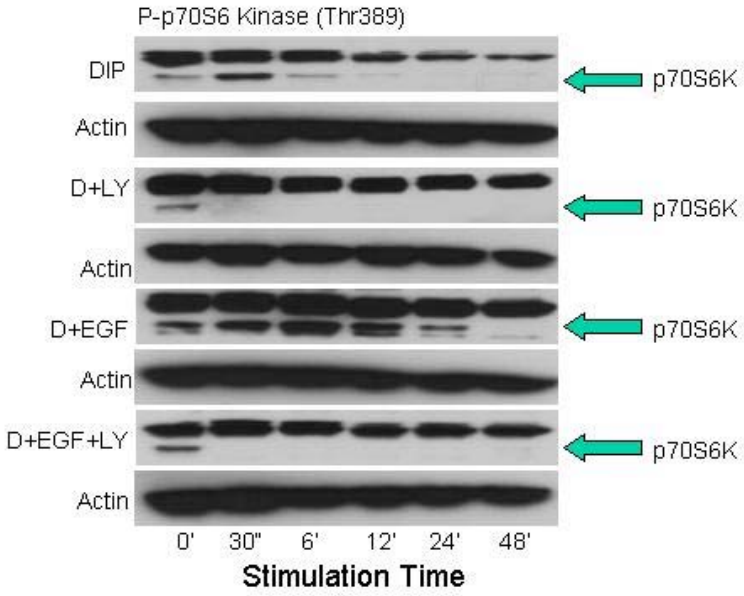
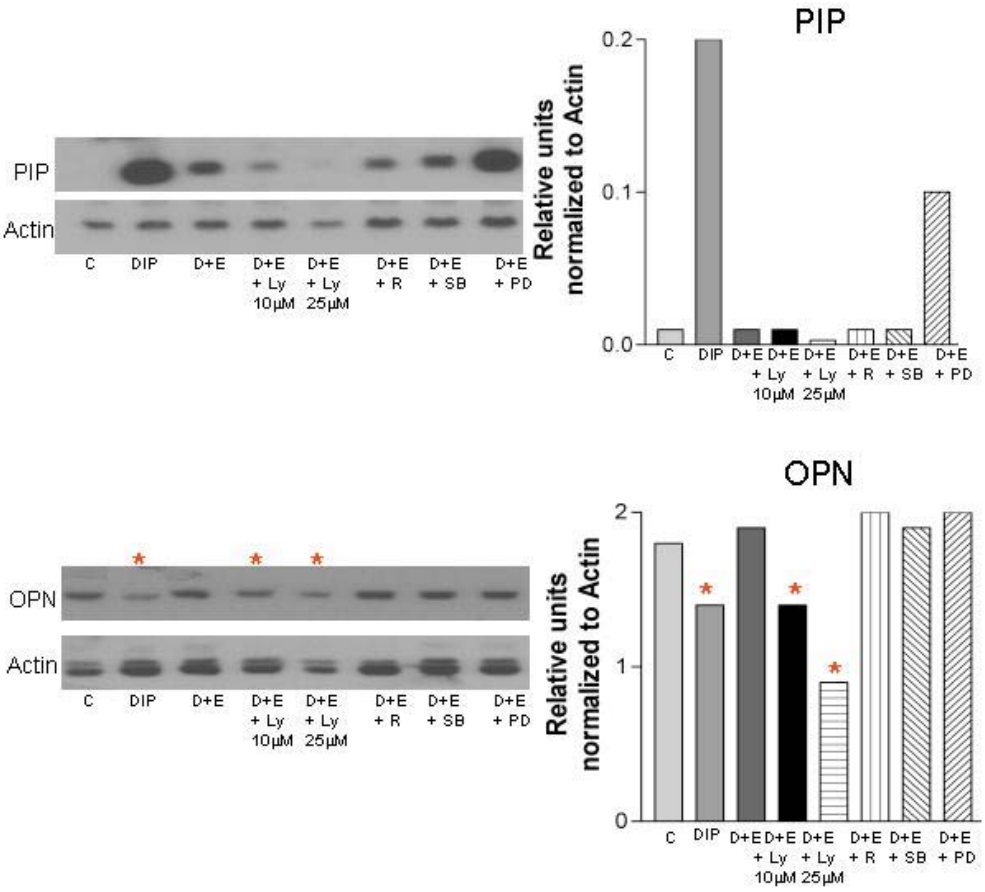

P-p70S6K (Thr389)
P-Akt (Ser473)
T-Akt
HA
p70S6K
Endogenous Akt
HA-CA-Akt
Endogenous Akt
HA-CA-Akt
0' 7.5' 15' 0' 7.5' 15'
C
CA-Akt
EGF Stimulation

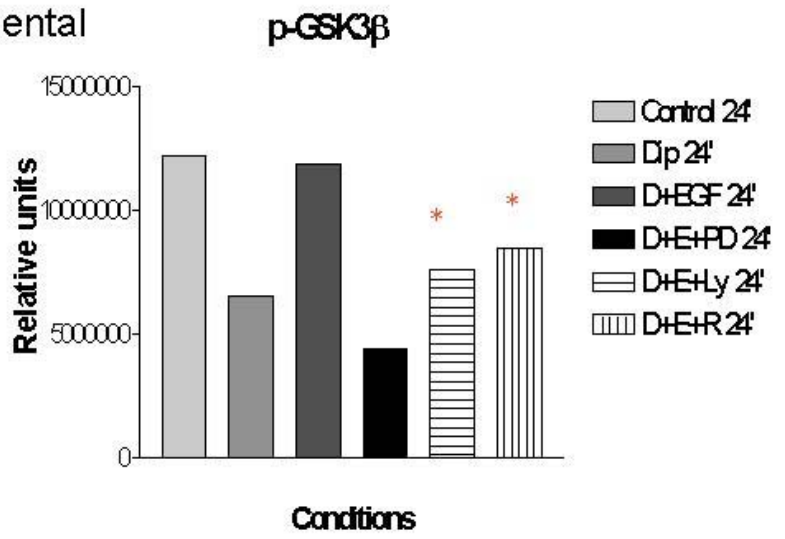
Figure 9

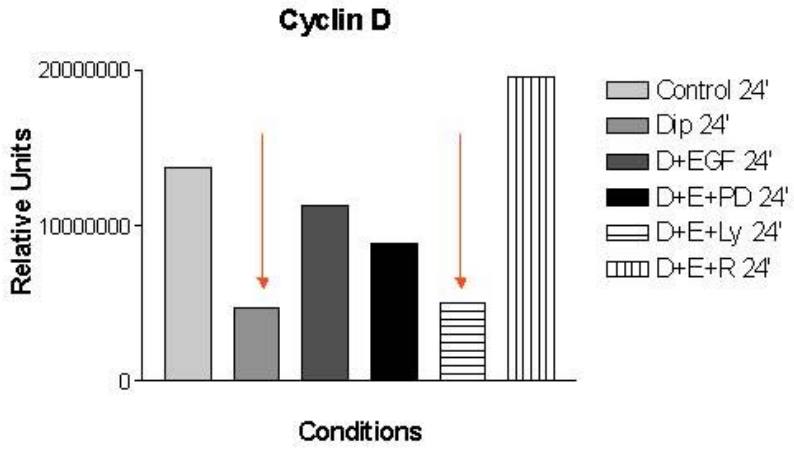

P-p70S6 Kinase (Thr389)
DIP
Actin
D+LY
Actin
D+EGF
Actin
D+EGF+LY
Actin
p70S6K
p70S6K
p70S6K
p70S6K
0'
30"
6'
12'
24'
48'
Stimulation Time

Supplemental Data



Supplemental Data





PART THREE

DISCUSSION

DISCUSSION

Mammary epithelial cells undergo episodic cycles of growth, differentiation, and apoptosis throughout a female's life. Mammary gland development can be divided into seven stages: embryonic, postnatal, juvenile, puberty, pregnancy, lactation, and involution. One of the leading risk factors for breast cancer is nullparity (Simpson, 2002). Without the induction of lactogenesis (terminal differentiation) followed by involution (apoptosis) a woman is more likely to develop breast cancer. Hence, the delineation of factors that inhibit lactogenesis is crucial to our understanding of breast cancer development.

Lactogenic differentiation is a complex process that involves the regulation of growth factors and hormones as well as epithelial--stroma interactions (Parmar, 2004). The hormones that influence lactogenic differentiation in mammary epithelial cells include prolactin and glucocorticoids such as dexamethasone. Many studies have been done to elucidate the involvement of prolactin in lactogenic differentiation as well as breast cancer development (Wang, 1992; Hankinson, 1999; Kabuto, 2000; Li, 2005; Hennighausen, 1997; Horseman, 1999). The involvement of dexamethasone in lactogenic differentiation is less understood; however, studies show that the removal of glucocorticoids such as dexamethasone and corticosterone induces involution (Feng, 1995; Berg, 2002).

The addition of growth factors that induce mitogenic pathways block mammary epithelial cell differentiation (Hynes, 1990; Merlo, 1996; Tonko-Geymayer, 2002; Petersen, 1998; Cerrito, 2004). Excess activation of signaling pathways downstream of the epidermal growth factor receptor (EGFR) has been linked directly to breast cancer

development and chemotherapeutic resistance (LeVea, 2004; Reise, 1998; Navolanic, 2003). While epidermal growth factor (EGF) is required for normal mammary epithelial cell proliferation, it has been shown to inhibit lactogenic differentiation of mammary epithelial cells both *in vitro* and *in vivo* (Hynes, 1990; Brandt, 2001; Cerrito, 2004). The two main signaling pathways activated by EGF stimulation in HC11 mammary epithelial cells are the Ras/Mek/Erk and the PI-3-K pathways (De Santis, 1997).

This thesis presents results from two papers aimed at elucidating the mechanisms by which Ras and PI-3-K block mammary epithelial cell differentiation. Our studies sought to add to the current knowledge of the mechanisms by which epidermal growth factor inhibits hormone-induced lactogenic differentiation in HC11 mammary epithelial cells. Our results demonstrate that EGF stimulation blocks lactogenic differentiation by Mek/Erk- and PI-3-K- dependent mechanisms. These results confirm the findings of previous studies that demonstrated that EGF blocked lactogenic differentiation in HC11 cells (Hynes, 1990; Brandt, 2001; Cerrito, 2004). In addition, our results confirm the findings that EGF activates Ras and PI-3-K in HC11 cells (De Santis, 1997). More specifically, we determined that blocking either one of these pathways by using either specific signal transduction inhibitors or by the use of either dominant negative (DN) or constitutively active (CA) forms of Ras or Akt, enhanced lactogenic differentiation both in the presence and absence of EGF. Moreover, the studies revealed that the activation of PI-3-K was independent of Ras, and that the Mek/Erk and PI-3-K pathways are synergistic in their action.

Prolactin is required for lactogenic differentiation and following binding to its receptor it causes Jak2 dimerization and auto-phosphorylation. Jak2 phosphorylates

Stat5A/B which then translocates to the nucleus where it binds to the β-casein promotor and activates its transcription. The SH2 protein tyrosine phosphatase, SHP2, has been identified in a complex with Stat5 and is proposed to regulate Stat5 activity (Berchtold, 1998; Chughtai, 2002). Our results indicated that DN-Ras expression blocked the association of SHP2 with Stat5 thereby enhancing Stat5 activation and lactogenic differentiation. In addition to involvement in Jak-Stat signaling, SHP2 is required for growth factor receptor activation of the Ras/Mek/Erk pathway. SHP2 plays an essential role in linking components of signal transduction pathways to growth factor receptor complexes via the scaffold protein Gab1, which targets SHP2 to the membrane (Cunnick, 2002). One potential link for SHP2 to the Ras pathway has recently been reported. Raynal et al. shows that an essential function of SHP2 is to down-regulate the interaction of Gab1 and Ras-GAP, thus allowing an efficient activation of Ras in response to EGF (Montagner, 2005). Our data demonstrated that DN-Ras expression, which interferes with Ras activation in part by binding and sequestering guanine nucleotide exchange factors (Lai, 1993), disrupts one aspect of SHP2 function. This suggests that there may be a mechanism to regulate SHP2 by Ras via SOS or RasGAP. In addition to Gab1, Gab2 has also been shown to recruit SHP2 to the membrane (Ali, 2000). Moreover, Gab2 increases proliferation via EGFR activation and the over-expression of Gab2 causes disorganized and defective mammospheres (Brummer, 2006). Thus, suggesting that excess Gab2 may play a role in blocking lactogenic differentiation through the activation of EGFR-dependent signaling.

While our study establishes two mechanisms by which the EGF-induced activation of Ras and PI-3-K blocks lactogenic differentiation other mechanisms have

been demonstrated. For example, EGF stimulation of HC11 cells was examined and shown to block lactogenic differentiation by increasing the levels of the phosphatase, PTP-PEST (Horsch, 2001). This increase in PTP-PEST directly correlated with a decrease in Jak2 phosphorylation. Another study revealed that EGF has a suppressive effect on Stat5 expression (Petersen, 1998).

The PI-3-K pathway is important in tumorigenesis in several ways. Aberrant PI-3-K activation has been demonstrated to promote both proliferation and survival of transformed cells, including those exhibiting EGF-dependent transformation (Lowe, 2004; Fry, 2001). The deregulation of many PI-3-K pathway components has recently been linked to a number of human malignancies (Vivanco, 2002; Fresno, 2004). Elevated Akt levels, for example, have been found in breast, ovarian, colon, and thyroid cancers (Lou, 2003; Vivanco, 2002). Comparisons of PI-3-K expression levels in breast tumor tissue from pre-menopausal versus post-menopausal patients demonstrate that the pre-menopausal tumors, noted for their associated poor prognosis and chemoresistance, express significantly higher levels of PI-3-K (Gershtein, 1999). The Yu laboratory demonstrated that the phosphorylation of Akt/mTOR increased progressively from normal breast epithelia to hyperplasia and abnormal hyperplasia to tumor invasion, suggesting that PI-3-K activity levels directly correlate with the degree of tumor progression (Zhou, 2004). These findings not only demonstrate the prominence of PI-3-K expression in breast cancer and its utility as prognostic indicator, but also lead to the speculation that ligand-induced and/or constitutive PI-3-K pathway activation might represent an important step in breast tumorigenesis.

Therefore, we investigated the role of proteins downstream of PI-3-K, in normal mammary epithelial cells, to elucidate the mechanism by which PI-3-K blocks lactogenic differentiation. We confirm that PI-3-K inhibits hormone-induced lactogenic differentiation in mammary epithelial cells (Merlo, 1996; De Santis, 1997). Two previous studies (Zeng, 2002; Xie, 2003) questioned whether PI-3-K activation of Akt in normal mammary epithelial cells is sufficient for cellular transformation. They concluded that cellular transformation is dependent on PDK1 activation and not Akt activation. Interestingly, we discovered that PDK1 is constitutively phosphorylated in HC11 cells and this is not blocked by LY294002. In addition, we demonstrated that PI-3-K activates Akt following EGF stimulation in HC11 cells. Moreover, CA-Akt blocks lactogenic differentiation and DN-Akt rescued HC11 cells from an EGF-induced inhibition of differentiation. This confirms that Akt plays a significant role in the PI-3-K pathways in HC11 cells. Several studies have also suggested that the activation of Erk is regulated through the PI-3-K pathway (Mahimainathan, 2005; Qiao, 2004), but our data demonstrates that EGF stimulation of Erk activation in HC11 mammary epithelial cells was not altered by blocking PI-3-K with LY294002. In addition, our previous work revealed that PI-3-K is activated directly by EGFR (ErbB1) and not by Ras (Cerrito, 2004). While no studies have addressed the mechanism by which PI-3-K blocks lactogenic differentiation, we demonstrated that the inhibition of PI-3-K, Akt, or mTOR blocked the activation p70S6 Kinase and its downstream targets. We also demonstrated that the expression of a CA-Akt leads to the constitutive activation of p70S6 Kinase. PDK1 has been shown to directly activate p70S6 Kinase independent of Akt, but our results suggest that the activation of p70S6 Kinase is dependent on Akt and mTOR in

HC11 cells (Pullen, 1998). These results suggest that the PI-3-K/Akt/mTOR pathway plays a significant role in inhibiting lactogenic differentiation.

Several ligands have been shown to bind to the EGFR (ErbB1) such as EGF, TGF-α, amphiregulin, and HB-EGF (Ram, 2000). EGF and TGFα bind to the EGFR and stimulate the proliferation of mammary epithelial cells and enhance lobular-aveolar development in the mammary gland of virgin mice (Vonderhaar, 1987). These growth factors can also prevent milk protein expression in HC11 cells and inhibit apoptosis of secretory alveolar epithelial cells in the involuting mammary gland (Smith, 1995). Transgenic studies also show a direct activation of the EGFR by TGF-α and that this activation is sufficient for induction of signal transduction pathways down-stream of the EGFR (Amundadottir, 1998; Humphreys, 2000). Interestingly, transgenic mice that over-express TGF-α display an increase in alveolar development, a marked delay in normal involution, and develop mammary tumors when compared to wild type mice (Troyer, 2001; Danielsen, 2001; Smith, 1995). Unfortunately, ErbB1-null mice only survive a short time postnatally, but there are several alternative transgenic models by which to study EGFR signaling. The first are female mice expressing a truncated EGFR that lacks a cytoplasmic sequence (Xie, 1997). The second are female mice harboring mutations that impair the EGFR tyrosine kinase activity (Luetteke, 1993). Both show impaired alveolar development and increased mammary tumorigensis (Troyer, 2001). Moreover, mammary glands from mice harboring an EGFR transgene under a control of the MMTV or β-lactoglobulin promotors developed abnormally and displayed mammary epithelial hyperplasia (Troyer, 2001).

Our studies have been extended to primary mouse mammary epithelial cells and we see the same inhibition of lactogenic differentiation by EGF as well as a rescue of this inhibition in the presence of the signal transduction inhibitors (unpublished data). Interestingly, there is new evidence emerging that suggests that the EGF receptor can translocate to the nucleus and affect proliferation as well as survival (Lo, 2005). Another study investigates EGF and involution by showing that the presence of EGF inhibits several genes (WDNM1, lactoferrin, lysozyme, ferritin heavy chain, and osteopontin) that are normally induced during this phase (Baik, 1998). While we have not investigated this in HC11 cells, we have some preliminary data that suggests Ras and PI-3-K inhibition during lactogenic differentiation cause apoptosis (unpublished data). In addition, a recent study has shown that expressing a CA-Ras (V12) in normal primary human mammary epithelial cells is sufficient in causing tumorigenesis (Kendall, 2005). There has also been direct evidence that Ras activation promotes tumor progression in mice that express an oncogenic version of Ras under the control of a MMTV promotor (Sinn, 1987; Andrechek, 2000). The expression of oncogenic Ras results in the induction of mammary tumors (Sinn, 1987).

Several *in vivo* Jak-Stat signaling models for studying lactogenic differentiation have also emerged. Stat5A and B knockout mice show impaired epithelial duct development as well as impaired lactogenesis (Teglund, 1998; Bromberg, 2000). In addition, Jak2 conditional knockout mice show an even more pronounced impairment of lactogenesis as compared to Stat5 knockout mice (Wagner, 2004). Moreover, two mouse models in which the phosphatases, CIS1 and SOCS1, that regulate Jak2-Stat5 activation are over-expressed or knocked out have been examined (Clevenger, 2003). The over-

expression of CIS1 and SOCS1 impairs mammary development and lactation. In contrast, the loss of CIS1 and SOCS1 accelerates mammary gland development during pregnancy. Each of these models has revealed an important role for Jak2-Stat5 activation for normal mammary epithelial cell differentiation. Hence, it would be interesting to extend our studies of EGF-signaling in these models.

Several studies demonstrate that the role of Ras and PI-3-K in cellular differentiation is tissue specific. For example, there is evidence from both *in vivo* and *in vitro* systems that the Ras/Mek/Erk pathway is required for neuronal differentiation (Halegoua, 1991; Marshall, 1995; Thomas, 1992; Wood, 1992). Moreover, PI-3-K activation promotes growth arrest in keratinocytes and adipocytes and has been demonstrated to be required for the differentiation of both (Calautti, 2005; Aubin, 2005). In intestinal epithelial cells, however, PI-3-K mediates proliferative signals and inhibits differentiation (Sheng, 2003). Hence, because of the dual nature of Mek/Erk and PI-3-K signaling in differentiation, it is important to understand the role of each pathway in lactogenic differentiation in mammary epithelial cells. Our conclusion is that Mek/Erk activation primarily blocks lactogenic differentiation by affecting Stat5 activation and transcription in mammary epithelial cells, and PI-3-K blocks lactogenic differentiation, in part, by regulating protein synthesis in mammary epithelial cells.

The results reported in this thesis establish mechanisms by which Ras and PI-3-K block HC11 lactogenic differentiation. However, further studies are required to address important questions that have yet to be answered. Additional studies are needed to more fully characterize the mechanism of action of dexamethasone during HC11 lactogenic differentiation. Also, a global translational expression pattern will need to be

performed to identify potential candidates regulated by PI-3-K. Preliminarily, we have shown that PI-3-K regulates cyclin D1 translation. SOCS1, 3 and CIS1 expression have also been examined and results reveal that PI-3-K does not seem to regulate the translation of these proteins. Other likely candidates would be the cyclin dependent kinase inhibitors p21 and p27 (Bader, 2005). Moreover, there is evidence that PI-3-K regulates the transcription of cyclin D1 and osteopontin (supplemental data). Thus, the investigation of PI-3-K activity and regulation of transcription needs to be addressed.

PI-3-K has also been reported to promote cellular survival through the inactivation of pro-apoptotic proteins like Bad and the activation of anti-apoptotic proteins like FKHR (Bader, 2005). Our preliminary data suggests that PI-3-K does not play a significant role in FKHR activation in HC11 differentiation. We believe this is due to the abundant production of extra-cellular matrix proteins by HC11 cells that stimulate cell survival signaling. However, more investigation is needed to elucidate the role of PI-3-K in cellular survival in HC11 differentiation. Lastly, the studies performed in HC11 cells will need to be reproduced in other normal mouse mammary epithelial cells lines, such as Eph4 cells, as well as primary mouse mammary epithelial cells.

In summary, physiological and epidemiological evidence suggests that early and numerous full-term pregnancies provide some degree of protection from breast cancer development because (1) mammary epithelial cells are fully differentiated during lactogenesis and then undergo apoptosis during involution allowing for cells containing damaged DNA to be eliminated from the pool of proliferating epithelial cells, and (2) the circulating hormone and growth factor changes during pregnancy result in down-regulation of key contributors to breast cancer development, *e.g.,* estrogen and epidermal

growth factor (Colditz, 2005; Dumitrescu, 2005). Therefore, the delineation of factors that inhibit lactogenesis is crucial to our understanding of the disease. We report the findings of two studies aimed at elucidating the mechanisms by which PI-3-K and Ras inhibit lactogenic differentiation in normal mammary epithelial cells. Many studies have addressed these pathways in cancer models (Gershtein, 1999; Yu, 2004; Ripple, 2005; Koziczak, 2004; Dunn, 2005), however, never fully elucidating the pathway during normal mammary differentiation. Hence, the understanding of normal signaling during lactogenic differentiation is important to our understanding of breast tumors that exhibit activated Ras and PI-3-K signaling. This understanding will ultimately lead to the development of breast cancer prevention techniques as well as therapeutic approaches in treating tumors where excessive activation of signaling pathways downstream of epidermal growth factor is detected.

PART FOUR

REFERENCES FOR INTRODUCTION AND DISSCUSSION

REFERENCES

(1996). "Breast cancer and hormonal contraceptives: collaborative reanalysis of individual data on 53 297 women with breast cancer and 100 239 women without breast cancer from 54 epidemiological studies. Collaborative Group on Hormonal Factors in Breast Cancer." Lancet 347(9017): 1713-27.

Ali, S. and S. Ali (2000). "Recruitment of the protein-tyrosine phosphatase SHP-2 to the C-terminal tyrosine of the prolactin receptor and to the adaptor protein Gab2." J Biol Chem 275(50): 39073-80.

Amundadottir, L. T. and P. Leder (1998). "Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes." Oncogene 16(6): 737-46.

Andrechek, E. R. and W. J. Muller (2000). "Tyrosine kinase signalling in breast cancer: tyrosine kinase-mediated signal transduction in transgenic mouse models of human breast cancer." Breast Cancer Res 2(3): 211-6.

Aubin, D., A. Gagnon, et al. (2005). "Phosphoinositide 3-kinase is required for human adipocyte differentiation in culture." Int J Obes (Lond) 29(8): 1006-9.

Bader, A. G., S. Kang, et al. (2005). "Oncogenic PI3K deregulates transcription and translation." Nat Rev Cancer 5(12): 921-9.

Baik, M. G., M. J. Lee, et al. (1998). "Gene expression during involution of mammary gland (review)." Int J Mol Med 2(1): 39-44.

Bailey, J. P., K. M. Nieport, et al. (2004). "Prolactin and transforming growth factor-beta signaling exert opposing effects on mammary gland morphogenesis, involution, and the Akt-forkhead pathway." Mol Endocrinol 18(5): 1171-84.

Beerli, R. R. and N. E. Hynes (1996). "Epidermal growth factor-related peptides activate distinct subsets of ErbB receptors and differ in their biological activities." J Biol Chem 271(11): 6071-6.

Berchtold, S., S. Volarevic, et al. (1998). "Dominant negative variants of the SHP-2 tyrosine phosphatase inhibit prolactin activation of Jak2 (janus kinase 2) and induction of Stat5 (signal transducer and activator of transcription 5)-dependent transcription." Mol Endocrinol 12(4): 556-67.

Berg, M. N., A. M. Dharmarajan, et al. (2002). "Glucocorticoids and progesterone prevent apoptosis in the lactating rat mammary gland." Endocrinology 143(1): 222-7.

Brandt, R., A. M. Wong, et al. (2001). "Mammary glands reconstituted with Neu/ErbB2 transformed HC11 cells provide a novel orthotopic tumor model for testing anti-cancer agents." Oncogene 20(39): 5459-65.

Bromberg, J. (2000). "Signal transducers and activators of transcription as regulators of growth, apoptosis and breast development." Breast Cancer Res 2(2): 86-90.

Brummer, T., D. Schramek, et al. (2006). "Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein." J Biol Chem 281(1): 626-37.

Calautti, E., J. Li, et al. (2005). "Phosphoinositide 3-kinase signaling to Akt promotes keratinocyte differentiation versus death." J Biol Chem 280(38): 32856-65.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science 296(5573): 1655-7.

Cantley, L. C. and B. G. Neel (1999). "New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway." Proc Natl Acad Sci U S A 96(8): 4240-5.

Cerrito, M. G., T. Galbaugh, et al. (2004). "Dominant negative Ras enhances lactogenic hormone-induced differentiation by blocking activation of the Raf-Mek-Erk signal transduction pathway." J Cell Physiol 201(2): 244-58.

Chughtai, N., S. Schimchowitsch, et al. (2002). "Prolactin induces SHP-2 association with Stat5, nuclear translocation, and binding to the beta-casein gene promoter in mammary cells." J Biol Chem 277(34): 31107-14.

Clevenger, C. V., P. A. Furth, et al. (2003). "The role of prolactin in mammary carcinoma." Endocr Rev 24(1): 1-27.

Colditz, G. A. (2005). "Epidemiology and prevention of breast cancer." Cancer Epidemiol Biomarkers Prev 14(4): 768-72.

Cunnick, J. M., S. Meng, et al. (2002). "Regulation of the mitogen-activated protein kinase signaling pathway by SHP2." J Biol Chem 277(11): 9498-504.

Danielsen, A. J. and N. J. Maihle (2002). "The EGF/ErbB receptor family and apoptosis." Growth Factors 20(1): 1-15.

De Santis, M. L., S. Kannan, et al. (1997). "Cripto-1 inhibits beta-casein expression in mammary epithelial cells through a p21ras-and phosphatidylinositol 3'-kinase-dependent pathway." Cell Growth Differ 8(12): 1257-66.

Dumitrescu, R. G. and I. Cotarla (2005). "Understanding breast cancer risk -- where do we stand in 2005?" J Cell Mol Med 9(1): 208-21.

Dunn, K. L., P. S. Espino, et al. (2005). "The Ras-MAPK signal transduction pathway, cancer and chromatin remodeling." Biochem Cell Biol 83(1): 1-14.

Earp, H. S., T. L. Dawson, et al. (1995). "Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research." Breast Cancer Res Treat 35(1): 115-32.

Feng, Z., A. Marti, et al. (1995). "Glucocorticoid and progesterone inhibit involution and programmed cell death in the mouse mammary gland." J Cell Biol 131(4): 1095-103.

Fresno Vara, J. A., E. Casado, et al. (2004). "PI3K/Akt signalling pathway and cancer." Cancer Treat Rev 30(2): 193-204.

Fruman, D. A., R. E. Meyers, et al. (1998). "Phosphoinositide kinases." Annu Rev Biochem 67: 481-507.

Fry, M. J. (2001). "Phosphoinositide 3-kinase signalling in breast cancer: how big a role might it play?" Breast Cancer Res 3(5): 304-12.

Gershtein, E. S., V. A. Shatskaya, et al. (1999). "Phospatidylinositol 3-kinase expression in human breast cancer." Clin Chim Acta 287(1-2): 59-67.

Hahn, W. C. and R. A. Weinberg (2002). "Modelling the molecular circuitry of cancer." Nat Rev Cancer 2(5): 331-41.

Halegoua, S., R. C. Armstrong, et al. (1991). "Dissecting the mode of action of a neuronal growth factor." Curr Top Microbiol Immunol 165: 119-70.

Hallmann, D., K. Trumper, et al. (2003). "Altered signaling and cell cycle regulation in embryonal stem cells with a disruption of the gene for phosphoinositide 3-kinase regulatory subunit p85alpha." J Biol Chem 278(7): 5099-108.

Hankinson, S. E., W. C. Willett, et al. (1999). "Plasma prolactin levels and subsequent risk of breast cancer in postmenopausal women." J Natl Cancer Inst 91(7): 629-34.

Hennighausen, L., G. W. Robinson, et al. (1997). "Prolactin signaling in mammary gland development." J Biol Chem 272(12): 7567-9.

Horsch, K., M. D. Schaller, et al. (2001). "The protein tyrosine phosphatase-PEST is implicated in the negative regulation of epidermal growth factor on PRL signaling in mammary epithelial cells." Mol Endocrinol 15(12): 2182-96.

Horseman, N. D. (1999). "Prolactin and mammary gland development." J Mammary Gland Biol Neoplasia 4(1): 79-88.

Humphreys, R. C. and L. Hennighausen (2000). "Transforming growth factor alpha and mouse models of human breast cancer." Oncogene 19(8): 1085-91.

Humphreys, R. C. and J. M. Rosen (1997). "Stably transfected HC11 cells provide an in vitro and in vivo model system for studying Wnt gene function." Cell Growth Differ 8(8): 839-49.

Hynes, N. E., D. Taverna, et al. (1990). "Epidermal growth factor receptor, but not c-erbB-2, activation prevents lactogenic hormone induction of the beta-casein gene in mouse mammary epithelial cells." Mol Cell Biol 10(8): 4027-34.

Kabuto, M., S. Akiba, et al. (2000). "A prospective study of estradiol and breast cancer in Japanese women." Cancer Epidemiol Biomarkers Prev 9(6): 575-9.

Kelly, P. A., A. Bachelot, et al. (2002). "The role of prolactin and growth hormone in mammary gland development." Mol Cell Endocrinol 197(1-2): 127-31.

Kendall, S. D., C. M. Linardic, et al. (2005). "A network of genetic events sufficient to convert normal human cells to a tumorigenic state." Cancer Res 65(21): 9824-8.

Koziczak, M., T. Holbro, et al. (2004). "Blocking of FGFR signaling inhibits breast cancer cell proliferation through downregulation of D-type cyclins." Oncogene 23(20): 3501-8.

Lai, C. C., M. Boguski, et al. (1993). "Influence of guanine nucleotides on complex formation between Ras and CDC25 proteins." Mol Cell Biol 13(3): 1345-52.

LeVea, C. M., J. E. Reeder, et al. (2004). "EGF-dependent cell cycle progression is controlled by density-dependent regulation of Akt activation." Exp Cell Res 297(1): 272-84.

Li, Y., C. V. Clevenger, et al. (2005). "Stabilization of prolactin receptor in breast cancer cells." Oncogene.

Lichtner, R. B. (2003). "Estrogen/EGF receptor interactions in breast cancer: rationale for new therapeutic combination strategies." Biomed Pharmacother 57(10): 447-51.

Lo, H. W., S. C. Hsu, et al. (2005). "EGFR signaling pathway in breast cancers: from traditional signal transduction to direct nuclear translocalization." Breast Cancer Res Treat: 1-8.

Lowe, W. L., Jr., R. Fu, et al. (1997). "Growth factor-induced transcription via the serum response element is inhibited by cyclic adenosine 3',5'-monophosphate in MCF-7 breast cancer cells." Endocrinology 138(6): 2219-26.
Luetteke, N. C., D. C. Lee, et al. (1993). "Regulation of fat and muscle development by transforming growth factor alpha in transgenic mice and in cultured cells." Cell Growth Differ 4(3): 203-13.

Luo, J., B. D. Manning, et al. (2003). "Targeting the PI3K-Akt pathway in human cancer: rationale and promise." Cancer Cell 4(4): 257-62.

Lynch, J. P. and D. G. Silberg (2002). "To differentiate or proliferate? The interaction between PI3K/PTEN and Cdx2." Gastroenterology 123(4): 1395-7.

Mahimainathan, L., N. Ghosh-Choudhury, et al. (2005). "EGF stimulates mesangial cell mitogenesis via PI3-kinase-mediated MAPK-dependent and AKT kinase-independent manner: involvement of c-fos and p27Kip1." Am J Physiol Renal Physiol 289(1): F72-82.

Marshall, M. S. (1995). "Ras target proteins in eukaryotic cells." Faseb J 9(13): 1311-8.

McPherson, K., C. M. Steel, et al. (2000). "ABC of breast diseases. Breast cancer-epidemiology, risk factors, and genetics." Bmj 321(7261): 624-8.

Merlo, G. R., D. Graus-Porta, et al. (1996). "Growth, differentiation and survival of HC11 mammary epithelial cells: diverse effects of receptor tyrosine kinase-activating peptide growth factors." Eur J Cell Biol 70(2): 97-105.

Montagner, A., A. Yart, et al. (2005). "A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation." J Biol Chem 280(7): 5350-60.

Navolanic, P. M., L. S. Steelman, et al. (2003). "EGFR family signaling and its association with breast cancer development and resistance to chemotherapy (Review)." Int J Oncol 22(2): 237-52.

Normanno, N., C. Bianco, et al. (2003). "Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment." Endocr Relat Cancer 10(1): 1-21.

Ohta, T. and M. Fukuda (2004). "Ubiquitin and breast cancer." Oncogene 23(11): 2079-88.

Parmar, H. and G. R. Cunha (2004). "Epithelial-stromal interactions in the mouse and human mammary gland in vivo." Endocr Relat Cancer 11(3): 437-58.

Petersen, H. and L. A. Haldosen (1998). "EGF modulates expression of STAT5 in mammary epithelial cells." Exp Cell Res 243(2): 347-58.

Pullen, N., P. B. Dennis, et al. (1998). "Phosphorylation and activation of p70s6k by PDK1." Science 279(5351): 707-10.

Qiao, M., P. Shapiro, et al. (2004). "Insulin-like growth factor-1 regulates endogenous RUNX2 activity in endothelial cells through a phosphatidylinositol 3-kinase/ERK-dependent and Akt-independent signaling pathway." J Biol Chem 279(41): 42709-18.

Ram, T. G., H. L. Hosick, et al. (2000). "Heregulin-beta is especially potent in activating phosphatidylinositol 3-kinase in nontransformed human mammary epithelial cells." J Cell Physiol 183(3): 301-13.

Richner, J., H. A. Gerber, et al. (1990). "c-erbB-2 protein expression in node negative breast cancer." Ann Oncol 1(4): 263-8.

Riese, D. J., 2nd and D. F. Stern (1998). "Specificity within the EGF family/ErbB receptor family signaling network." Bioessays 20(1): 41-8.

Ripple, M. O., S. Kalmadi, et al. (2005). "Inhibition of either phosphatidylinositol 3-kinase/Akt or the mitogen/extracellular-regulated kinase, MEK/ERK, signaling pathways suppress growth of breast cancer cell lines, but MEK/ERK signaling is critical for cell survival." Breast Cancer Res Treat 93(2): 177-88.

Sarbassov, D. D., D. A. Guertin, et al. (2005). "Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex." Science 307(5712): 1098-101.

Shen, Q. and P. H. Brown (2003). "Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways." J Mammary Gland Biol Neoplasia 8(1): 45-73.

Sheng, H., J. Shao, et al. (2003). "Phosphatidylinositol 3-kinase mediates proliferative signals in intestinal epithelial cells." Gut 52(10): 1472-8.

Shtiegman, K. and Y. Yarden (2003). "The role of ubiquitylation in signaling by growth factors: implications to cancer." Semin Cancer Biol 13(1): 29-40.

Simpson, H. W., C. S. McArdle, et al. (2002). "Pregnancy postponement and childlessness leads to chronic hypervascularity of the breasts and cancer risk." Br J Cancer 87(11): 1246-52.

Sinn, E., W. Muller, et al. (1987). "Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo." Cell 49(4): 465-75.

Smith, G. H., R. Sharp, et al. (1995). "Transforming growth factor-alpha promotes mammary tumorigenesis through selective survival and growth of secretory epithelial cells." Am J Pathol 147(4): 1081-96.

Stern, D. F. (2003). "ErbBs in mammary development." Exp Cell Res 284(1): 89-98.

Teglund, S., C. McKay, et al. (1998). "Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses." Cell 93(5): 841-50.

Thomas, S. M., M. DeMarco, et al. (1992). "Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases." Cell 68(6): 1031-40.

Tonko-Geymayer, S., O. Goupille, et al. (2002). "Regulation and function of the cytokine-inducible SH-2 domain proteins, CIS and SOCS3, in mammary epithelial cells." Mol Endocrinol 16(7): 1680-95.

Troyer, K. L. and D. C. Lee (2001). "Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network." J Mammary Gland Biol Neoplasia 6(1): 7-21.

Veronesi, U., P. Boyle, et al. (2005). "Breast cancer." Lancet 365(9472): 1727-41.

Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." Nat Rev Cancer 2(7): 489-501.

Vonderhaar, B. K. (1987). "Local effects of EGF, alpha-TGF, and EGF-like growth factors on lobuloalveolar development of the mouse mammary gland in vivo." J Cell Physiol 132(3): 581-4.

Wagner, K. U., A. Krempler, et al. (2004). "Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice." Mol Cell Biol 24(12): 5510-20.

Wang, D. Y., B. L. De Stavola, et al. (1992). "Relationship of blood prolactin levels and the risk of subsequent breast cancer." Int J Epidemiol 21(2): 214-21.

Wartmann, M., N. Cella, et al. (1996). "Lactogenic hormone activation of Stat5 and transcription of the beta-casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity." J Biol Chem 271(50): 31863-8.

Wood, K. W., C. Sarnecki, et al. (1992). "ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK." Cell 68(6): 1041-50.

Xie, W., A. J. Paterson, et al. (1997). "Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development." Mol Endocrinol 11(12): 1766-81.

Xie, Z., X. Zeng, et al. (2003). "Transformation of mammary epithelial cells by 3-

phosphoinositide- dependent protein kinase-1 activates beta-catenin and c-Myc, and down-regulates caveolin-1." Cancer Res 63(17): 5370-5.

Yu, X., R. V. Rajala, et al. (2004). "Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17 beta-estradiol-mediated neuroprotection." J Biol Chem 279(13): 13086-94.

Zeng, X., H. Xu, et al. (2002). "Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with the induction of protein kinase Calpha." Cancer Res 62(12): 3538-43.

Zhou, X., M. Tan, et al. (2004). "Activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway by ErbB2 overexpression predicts tumor progression in breast cancers." Clin Cancer Res 10(20): 6779-88.